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CONTENTS

ORIGIN OF MELANOPHORES AND THEIR ROLE IN DEVELOPMENT OF COLOR PATTERNS IN VERTEBRATES

Mary E. Rawles 383

ANTICONVULSANTS

James E. P. Toman and Louis S. Goodman 409

GASTRIC ABSORPTION

Leonard Karel 433

THE PHYSIOLOGY OF ADIPOSE TISSUE

E. Wertheimer and B. Shapiro 451

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BALDUIN LUCKÉ: Neoplasia in Cold-Blooded Vertebrates

B. H. WILLIER: The Production of Pigmentation Patterns in Birds

HALLOWELL DAVIS: Recent Advances in the Physiology of Audition

HAROLD SPEERT: Local Action of Sex Hormones

(Continued on cover 4)

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ORIGIN OF MELANOPHORES AND THEIR RÔLE IN DEVELOPMENT OF COLOR PATTERNS IN VERTEBRATES

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IT IS PROBABLE that no phenomenon of nature has attracted more attention over a longer period of time or has been investigated from more diverse points of view than that of pigment formation in the integument of animals. Among the vertebrates, color patterns may be produced in a number of different ways, but in the majority of cases melanin pigment is the principal color constituent. Although melanin pigment-forming cells have been known and described for more than a century, it is only within recent years that the fundamental question of the embryonic origin of these cells has been definitely settled within three groups—amphibians, birds and mammals. It is only through the knowledge that the pigmentary unit, the melanophore, has an extrinsic origin in the neural crest that it has become possible to apply experimental methods of causal analysis towards discovering the mechanisms of control in the development of the definitive system of pigmentation. Through such analysis, definite progress is being made towards an understanding of the complex interrelation of factors, genetic and environmental, involved in the development of the final color pattern.

The main purpose of the present article is to bring together in one summary the principal findings obtained so far from the work on amphibians, birds and mammals, which quite definitely are leading to a more unified concept of the rôle of the melanophore in the development of pigmentation in the vertebrates in general.

MELANIN PIGMENTS

The melanin pigments are produced in certain highly specialized, branched, migratory cells which bear a striking morphological similarity in all of the vertebrates. These pigment-forming cells have been designated by a variety of names—chromatophores, melanophores, melanoblasts, pigmentoblasts, dendritic cells—which has led to some confusion and misunderstanding. Some authors have, therefore, resorted to referring to them simply as pigment-cells. In the following pages the

terms melanophore and pigment-cell are used interchangeably to designate cells containing melanin granules which they themselves have synthesized; the term melanoblast is used to designate an undifferentiated, prospective melanophore or pigment-forming cell.

The melanin pigments produced by the melanophores are granular in nature, the granules being of definite shape and measurable size, ranging in color from pale yellow or buff through various shades of red-brown to black. Numerous histological studies of the definitive granules deposited in the feather cells of a variety of birds (106, 71, 63, 44, 87, 126, 17) and in the hair cells of various mammals (123, 103, 49, 26, 100) have revealed that within any one genotype, the size, shape and color of the granules exhibit a remarkable specificity. In general, the dark melanins occur in the form of rods, the light melanins in the form of spheres. Also, the dark colored granules are much less soluble in alkalis than those of lighter color, probably due to differences in degree of oxidation of the melanin precursors (44). Undoubtedly the so-called diffuse melanins mentioned in hair and feather studies, time and again by the older workers in particular, can be accounted for on the basis of partial solubility of the granules which occurred during the preparation of the material for microscopic study. According to the present status of our knowledge, melanin exists only in the form of discrete particles.

Melanin granules have usually been considered as inert deposits of the end product of cellular metabolism. That they can no longer be considered as such has been demonstrated recently by the work of Hermann and Boss (54) with pigment granules isolated from bovine ciliary bodies by differential centrifugation. Examination of these isolated melanin granules for certain enzymatic components has revealed the presence of three oxidative enzymes—dopa oxidase, cytochrome oxidase and succinic-dehydrogenase.

One of the most important problems in melanin pigmentation centers around the mechanism involved in the formation of the characteristic and uniformly shaped granules formed within a single melanophore. Ever since Reinke in 1894 (97) pointed out clearly that the melanin granules are composite structures made up of a colorless substrate, Pigmentgrundsubstanz or Pigmentträger, to which the coloring matter melanin is attached, numerous statements have been made in the literature regarding their origin. It was natural that the early investigators, in view of the importance assigned to the cell nucleus at the time, should seek to identify the necessary morphological ground substance (Pigmentträger or Pigmentbildner) with the nuclear chromatin elements. The chief exponent of this view was Aurel von Szily (117), who in 1911 published an extensive monograph on the subject. He described colorless bodies (Pigmentträger) extruded from the nucleus and derived directly from its chromatic elements, which, after wandering to a more or less peripheral position in the cell, became colored by the action of cell ferments. Others tried to derive the Pigmentträger from filaments of the protoplasm (38, 62) and Kromayer (62), thought he could observe a direct transition of protoplasmic fibers to pigment granules in epithelial cells. Later on, as various cytoplasmic constituents became investigated, these elements, particularly the mitochondria (95, 59) and the lipochondria (42, 74) were considered to be the morphological constituents from which the de-

finitive pigment granules arose. The diversity of opinion was no doubt due to the fact that all of the conclusions were drawn merely from fixed and stained material which varied considerably depending upon the techniques applied. Interestingly enough, with the discovery in recent years of submicroscopic particles of cytoplasmic origin and of highly complex chemical composition (phospholipids and ribonucleoproteins) by Bensley (7, 8), Claude (20, 21) and others, attention is now being focused upon them as possible sources of origin of melanin pigment granules (61).

The chief difficulty in formulating an hypothesis to account for the formation of melanin granules centers in the fact that the chemical composition of melanin is not known. In fact it is quite probable that all of the substances to which the term 'melanin' has been applied are not identical (77). It has been generally accepted since the discovery of tyrosinase by Bertrand, in 1896 (9), that a substrate or melanogen and an oxidative enzyme are involved in melanin synthesis; oxidation products resembling natural melanins have been demonstrated in many animals and plants. Histological studies on human and other mammalian skin led Bloch (15, 16) to the assumption that melanin is formed by a highly specific enzymatic oxidation of 1-dihydroxyphenylalanine (dopa). The enzyme became known as dopa oxidase. While Bloch's results have been substantiated by many workers the specificity attributed by him to dopa oxidase has met with severe criticism (see Franke (45) for a short summary). The criticism is based mainly on the contention that the ease with which the reaction occurs may depend on the ease with which dopa is known to oxidize rather than on the specificity of the enzyme present. The experimental results of Figge (41) suggest strongly that in certain amphibians melanin pigment formation is the result of the oxidation of tyrosine by tyrosinase and is regulated by a delicate balance of oxidative and reductive mechanisms within the melanophores.

In recent years Hogeboom and Adams (55) working with cell-free extracts of melanotic mouse tumors, which presumably arose from skin pigment cells, obtained two highly specific fractions: one containing an enzyme which catalyzes tyrosine to melanin (tyrosinase), another which catalyzes dihydroxyphenylalanine to melanin (dopa oxidase). Also, as previously mentioned, Hermann and Boss (54) have demonstrated the activity of dopa oxidase in purified melanin granules of bovine ciliary bodies. Their purified preparation failed to oxidize tyrosine, hydroquinone and catechol unless cytochrome *c* was added. The important point in this respect, however, is the fact that dopa could be oxidized to form melanin pigment without the added cytochrome *c*. These results indicate that dopa may be oxidized *in vitro* by at least two different enzyme systems, one of which is concerned primarily in the formation of melanin. The distribution of cytochrome-*c*, cytochrome oxidase and dopa oxidase as well as the relative reducing intensity of the cells appears to be of importance in the formation of melanin pigment. From numerous investigations but particularly those of Hogeboom and Adams, and Hermann and Boss, it appears quite probable that the oxidation of dopa through a system other than that of the generally distributed cytochrome oxidase system is the important reaction in melanin formation. If these interesting findings can be verified in other melanin pigment-forming tissues, we shall have gone a long way towards an under-

standing of the complicated problem of melanogenesis. It is of interest to note that in both of the aforementioned investigations the experimental material was relatively homogeneous and extremely rich in pigment. In the light of these findings, the failure heretofore to demonstrate either tyrosinase or dopa oxidase in mammalian skin extracts could very well be attributed to the relatively small quantity of the enzyme present with respect to the total volume of tissue concerned.

In view of the fact that during the past few years it has become increasingly evident that the submicroscopic granules (and the mitochondria) are seats of enzymatic activity and the fact that melanin granules of the ciliary body have already been shown to contain several enzyme systems, including dopa oxidase, it may not be going too far afield to suggest that the submicroscopic granules are the sites of melanin synthesis. Assuming that these specialized particulates in the cytoplasm of a prospective melanophore (melanoblast) contain the specific enzyme for the oxidation of the melanin precursor, the ultimate melanin granules could be built up by an accumulation and subsequent polymerization of the oxidation products; the specific size, shape etc. depending upon the nature of the polymer. (See Kopac (61) for further discussion in this new and interesting direction.) With the progress that is now being made in protein and cytochemistry we have every reason to believe that the time is not too far distant when we shall have a satisfactory solution to the as yet little understood process of melanin synthesis.

EMBRYONIC ORIGIN OF THE MELANOPHORES

In order to arrive at some understanding of the interplay of factors, genetic and environmental, which are necessarily involved in the development of a system of pigmentation, it is of the utmost importance to know the source of the pigmentary unit, the melanophore. For nearly one hundred years a wide variety of histological observations on embryonic and adult tissues of every class of vertebrates has been brought to bear upon the general problem of melanophore origin. The theories which have been advanced have been reviewed many times. See Post (84), von Szily (117), Fuchs (46), Biedermann (10–12), Strong (109) and more recently DuShane (32, 33). At the present time, it is so clearly established that the neural crest is the source of all pigment cells (exclusive of the retinal tapetum) in amphibians, birds and mammals that other hypotheses need no longer be considered.

The composite nature of the neural crest is well known. Cells destined to give rise to a variety of tissues, in addition to the pigment cells, such as visceral cartilages, ganglia and sheath cells have been shown by Harrison (51), Stone (108), Horstadius and Sellman (58), Adelmann (1), Holmdahl (56) and others to take their origin from the crest. In their early developmental stages the prospective pigment cells (melanoblasts) cannot be distinguished either morphologically or histologically from the other embryonic cells with which they are associated. They are at first without pigment and often reach remote positions in the body of the embryo before showing their characteristic differentiation. For this reason their true origin cannot be ascertained by the study of serial sections alone—the method used almost exclusively until recent years. With the introduction and perfection of precise techniques, by Spemann and his school, for isolating and transplanting regions of the embryo, it

became possible to analyze the inherent developmental capacities of embryonic tissues. It was primarily through the use of such methods that the neural crest origin of pigment cells was, finally, clearly demonstrated.

The amphibians were the first group in which information was obtained for linking pigment cell origin with the crest, and the evidence was gradually accumulated. Harrison (50), in his study of the growth of nerve fibers in tissue cultures, noted that pigmented cells frequently formed from pieces of medullary cord. He thought their origin from the ganglion crest likely. Later Mangold (75) and others and Holtfreter (57) obtained evidence from various transplantation experiments with Triton embryos, which pointed strongly to a neural crest origin of the pigment cells. Since the primary interest of these investigators was directed along lines other than those of pigmentation, it remained for other experimentalists to furnish the conclusive proof.

DuShane (31), working with Amblystoma, found that removal of the neural folds (the primordia of the neural crest) in the neurula stage resulted later in a total lack of pigment cells in the operated (trunk) region; and the isolated folds produced numerous pigment cells when grown in culture medium or transplanted to the ventral region of another embryo. After closure of the neural folds, in tail-bud stages, the extirpated crest region transplanted heteroplastically between two pigmented embryos invariably produced pigment cells of the donor type. Similar observations were made by Twitty (111) and Twitty and Bodenstein (114) on several species of Triturus and by Raven (86) on Triton, and confirmed many times by subsequent workers not only for the crest of the trunk region but also for that of other body regions (82). Bytinsky-Salz (18) and Baltzer (3) extended the investigations to include the anurans. By means of xenoplastic transplantations, they were able to show that the neural crest of the head and trunk regions gives rise to all types of pigment cells, guanophores as well as melanophores. Also, Barden (5) working with the amphibian eye demonstrated conclusively that the xanthophores, guanophores and melanophores found in the chorioidea and iris arise from the neural crest. For a time there was some question among amphibian workers as to whether the melanophores found in the epidermis, which become very conspicuous at metamorphosis and play the major rôle in the pigmentation of the adult in certain species, were also of neural crest origin. The experiments of Rosin (99) as well as the recent experiments of Stearner (107), in which special consideration was given to this question, would seem to leave no further doubt that in the urodeles the epidermal melanophores do originate in the neural crest. Stearner's observations have shown further that these melanophores are the sole source of the melanin pigment granules which are deposited in the epidermal cells. At the present time, then, the large body of evidence agrees in showing that the neural crest is the source of all types of pigment cells—melanophores, xanthophores, guanophores—that develop in various parts of the body of an amphibian. While the crest as a whole is potentially capable of producing pigment cells it does, however, exhibit conspicuous differences along its cranio-caudal extent with respect to the relative number of pigment cells produced; the crest of the trunk region is by far the richest source (82).

In birds, the evidence pertaining to the origin of melanophores follows similar

lines. Dorris (29) found that explants including the neural crest of the pre-otic region of early chick embryos of several breeds produced typical melanophores in culture medium. Later (30) she showed that similar isolates transplanted to the leg bud of three-day host embryos produced donor coloration in the down feathers of the host leg (cf. 34, 36, 126). Ris (98), working with intracoelomic and other grafts from various axial levels of embryos of several varieties of fowl and other birds, was able to correlate the presence of pigment cells in the grafts with the morphological development of neural crest at the time the isolation was made. Thus, it was conclusively demonstrated that only those isolates known to contain neural crest, the region from which it arises, or its migratory cells, are capable of producing melanophores in grafts. So reliable has this correlation proved that pigment-free (white) feathers can be produced, at will, from any potentially pigmented variety of bird by the simple precaution of excluding neural crest cells from the desired region (90). In an appropriately designed experiment, Ris (98) was also able to determine the origin of the retinal and chorioidal pigments of the eye in fowl. The retinal pigment does not occur in branched cells. It arises *in situ*. The pigment of the chorioidea (and iris) is, however, produced in characteristically branched cells, melanophores, identical with those in the skin and mesoderm and, like them, derived from the neural crest. It is worth mentioning that pigment cells comparable to xanthophores, guanophores, liphores etc. in amphibians do not exist in birds. The important and widely occurring lipochrome pigments found within the feather cells of birds are not produced by specialized migratory pigment-forming cells. These pigments are diffuse rather than granular and appear to be of exogenous (plant) origin. They are dissolved in the fat droplets deposited inside of the cells of the barb ridges prior to the onset of keratinization (28, 116).

The only mammalian embryo in which the origin of melanin pigment-forming cells from the neural crest has actually been demonstrated is the mouse (89, 92). The experimental procedure was similar to that used for demonstrating the origin of melanophores in birds, namely, the isolation of tissues from various axial levels at different developmental stages and their subsequent transplantation to the embryonic coelom of White Leghorn chick hosts. Data obtained from an extensive series of experiments with embryos of a homozygous black strain of mouse have demonstrated, clearly, that only those tissues containing presumptive neural crest, histologically recognizable neural crest or cells migrating from the neural crest, can produce melanophores. The fact that well-developed, structurally normal hairs entirely without pigment (white) can be obtained from a potentially pigmented (black) strain of mouse, if certain body regions are grafted at a particular developmental stage, shows very definitely that the ectoderm and its derivatives, the hairs, have no inherent capacity for autonomous melanin-pigment formation.

In the remaining vertebrate groups, the fishes and the reptiles, the origin of melanophores has received little attention in spite of the fact that in both of these classes of animals melanophore activity has been investigated intensively with respect to color changes. (See Parker (83) for a survey of the investigations on animal color changes from the years 1910 to 1943.) From the recent transplantation experiments of Lopashov (73) on teleost embryos, it is evident that the melanophores

are derived from the anlage of the central nervous system. It is highly probable that melanophore origin in fishes and in reptiles, also, is linked with neural crest differentiation.

While there is no doubt that in the vertebrates investigated the pigment-forming cells arise from the neural crest, it should be pointed out that the organization of the crest as an embryonic structure is, as yet, little understood (53). It is not known, for example, whether the cells of the crest are pluripotent from the beginning and differentiate according to their later positions or whether their potentialities are already fixed before migration begins. The question of cell differentiation is one of the most fundamental and at the same time one of the most obscure problems of all biology. The neural crest would seem to offer especially favorable material for a study of the differentiation of embryonic cells into specific cell types.

MIGRATION OF MELANOPHORES

Having demonstrated that melanophores originate in the neural crest, the question next arises as to how and when they reach those parts of the body, the skin and its derivatives, visceral membranes etc., in which they are later found. Conclusive evidence that prospective pigment cells (melanoblasts) reach their destinations in the feather germs of birds by migration has been obtained by implanting small pieces of embryonic tissue containing neural crest cells in the early wing buds of host embryos of a different breed (126). At hatching, the plumage of the entire host wing, and often adjacent regions of the breast, invariably exhibit the color pattern characteristic of the donor individual. Furthermore, the melanin granules deposited by the grafted pigment cells in the host feathers are always of the size, shape and color characteristic of the donor breed, even when the donor represents another order of birds (87).

In the absence of suitable cytological criteria for recognizing pigment cells in their early unpigmented stages, their exact migratory pathways are not known. It has been possible, however, to ascertain with a fair degree of accuracy the direction of migration and the time when these cells reach their definitive locations in both chick and mouse embryos. The general procedure has been to isolate systematically various portions of the embryo from different axial levels at successive developmental stages (during somite formation) and to test the capacity of such isolates for pigment cell production in grafts. For example, if the forelimb bud is removed from a 9- to 10-day mouse embryo of a homozygous black strain and allowed to continue its development in a site, such as the coelom of a White Leghorn chick embryo, known to be favorable for further growth and differentiation, it will produce hairs entirely without pigment (white). If taken a little later, the identical isolation will produce pigmented (black) hairs. In the interval of time between the two isolations, prospective pigment cells have migrated into the limb bud, as evidenced by the presence of pigment in the hairs (89, 92). Similarly, the wing bud of a potentially pigmented chick embryo will produce white feathers if isolated before approximately 80-hours' incubation; pigmented feathers after that time (34, 98). The hind limb bud will not produce pigmented feathers until still later, 96 to 103 hours etc. (120, 126).

Thus by testing not only limb buds, but somites, skin ectoderm, mesoderm, neural tube etc., alone and in combination, it has been possible to demonstrate 1) that

the direction of migration of melanoblasts is mediolateral and 2) that melanoblasts have reached their definitive locations in all parts of a chick embryo by the 4th day of incubation and in all parts of a mouse embryo by the 12th day of gestation.

In amphibians, the experimentation has been carried out from a somewhat different point of view. Nevertheless, it is evident from the transplantations of DuShane (31) that melanoblasts have reached their definitive locations in the limb buds by the late tail bud stage and, from the experiments of Twitty (111) and Twitty and Bodenstein (115), it is clear that in normal development melanoblasts show a general tendency to migrate in a medio-lateral direction, just as they do in birds and mammals.

That pigment cells migrate extensively in their early, unpigmented stages is quite evident from the foregoing account. In birds there is no evidence that a fully differentiated (pigmented) melanophore can shift its position to any great extent, even when grown *in vitro* (47). In amphibians the relationship is not necessarily so rigid, at least when the cells are cultivated under certain conditions *in vitro* (112, 113). However, the observed movements initiated after the onset of melanin granule formation in cultures are by no means comparable to the extensive migrations occurring normally when these cells are in their unpigmented phases.

It would be important to know the nature of the factors which influence the pathways of migration. Are they intrinsic to the pigment cells themselves or are they extrinsic, i.e. imposed by the tissue environment, or both?

Intrinsic factors have been demonstrated in amphibians (114, 115, 112) and in birds (126). Melanoblasts of certain species of amphibian embryos exhibit a marked inherent tendency to spread more extensively under identical experimental conditions *in vivo* and *in vitro*, than those of other species, and among the white varieties of fowl, White Leghorn melanoblasts show far less ability to migrate when implanted into the wing bud of a pigmented host than melanoblasts from any of the so-called recessive white varieties (Wyandotte, Plymouth Rock, Silkie).

Among extrinsic factors affecting the migratory activities of grafted melanoblasts is the age of the host embryo. Melanoblasts from a three-day chick donor embryo implanted to the wing bud of an older (4–5 days) host, after the host's neural crest cells have already migrated into the wing bud, as a rule do not leave the implant to produce the extensive area of donor-coloration in the plumage of the wing of the host, which is observed when younger hosts are used (126). Likewise, the migration of melanoblasts from transplanted neural crest in amphibians is much less extensive in older than in younger hosts (115).

Other conditions in the host, apart from age, have been shown to influence migration. A conspicuous example is found in Triturus (111). Whenever *T. torosus* is host to either *T. rivularis* or *T. simulans*, the grafted melanophores do not descend below the dorsal margin of the yolk mass of the host while in their normal environments or in other host environments they do not stop at this point. Even more pronounced examples of host influence on grafted melanophores may be found when donor and host represent different orders. Under the influence of Hyla hosts, Triton melanoblasts surpass their normal migratory boundaries and become distributed in the ventral body region (2, 4, 64). In birds there is, also, good evidence that consti-

tutional differences in hosts may either favor or restrict the extent of migration of donor melanoblasts introduced into the wing bud (126).

The extent of the area of donor-colored plumage, hence the degree of pigment cell migration, in wing bud grafts is controlled to a great extent by the quantity or concentration of melanoblasts originally introduced. Similar observations have been made in amphibians, particularly by growing neural crest cells in cultures under especially devised experimental conditions (112). In fact, Twitty considers this observed quantitative relationship between size of cell colony (population density) and extent of migration important supporting evidence for his hypothesis that migration is a response to mutual influences exerted by the melanoblasts themselves through the release of metabolic substances. According to this conception, melanoblasts would continue to move away from their neighbors until spaced beyond the effective range of influence. Hence, the larger the original population the greater the distance travelled by the individual cells in achieving such spacing.

The possibility of purely mechanical hindrances to the movements of melanoblasts should not be entirely overlooked. In birds there is good evidence that a sudden change in the arrangement of cells in adjacent tissues can effectively block melanoblast entry (98, 121, 90); and, with the failure of development of the dorsal fin in amphibians, melanoblasts, which normally stop at this point, now migrate freely across the mid line (113). It is quite probable that the accumulation of melanoblasts at the margin of the yolk mass in certain species of Triturus and in Triton belongs in this category.

While it is evident that pigment cells are capable of independent movements, much of their migratory activity is unquestionably dependent upon their contact with certain other cell strains and tissues. The fact that melanophores are found primarily along surface membranes, e.g. the basal layer of the epidermis, parietal membranes, meninges, walls of blood vessels, walls of sex and urinary ducts etc., is significant. (For a discussion bearing upon the problems of selective cell association, tissue affinities etc., the reader is referred to a recent paper of Weiss (122) dealing with the general problem of cell specificity in growth and development.) The possibility that the final distribution of melanophores may be affected by morphogenetic movements and by growth movement of their tissue substrates must be considered. Watterson (121), in his studies on the development of the feather germ, describes a succession of changes in melanophore distribution which are definitely correlated with morphogenetic movements of the epidermal cells in the organization of barb ridges. Among amphibian workers it appears to be rather generally accepted that melanophores of the larva can be carried along passively, to some extent, by the rapid growth of their tissue substrates (99, 113). Other aspects of pigment-cell migration in relation to pattern formation will be considered in the following section.

INTERACTION OF MELANOPHORES AND TISSUE ENVIRONMENT IN THE FORMATION OF DEFINITIVE PIGMENTARY PATTERNS

Most of our knowledge concerning the development of patterns of pigmentation in the vertebrates has come in recent years through experimental studies in two widely different groups, amphibians and birds. As the investigations have gradually

broadened it has become more and more evident that the underlying principles involved are remarkably similar in both groups. In the analyses of pattern formation, both in the embryo and in the adult animal, the investigations have centered around two fundamental problems: *a*) the part played by the individual pigment cells and *b*) the part played by the tissues with which the pigment cells become associated. Both, as we shall see, figure importantly in the formation of the definitive pigmentary pattern.

Larval Amphibian

Among larval amphibians there is a general tendency for the melanophores to be either uniformly distributed over the dorsal and lateral surfaces of the somites or confined more or less sharply into bands or stripes which have a definite relation to certain regions. These observed differences in melanophore distribution do not necessarily reflect corresponding differences in the original dispersal of prospective melanophores from the neural crest. The most recent evidence indicates that in Triton and Triturus the characteristic longitudinal stripes are formed by a secondary rearrangement of melanophores, originally more widely scattered over the lateral surfaces of the somites (99, 113). Aside from characteristic differences in melanophore arrangement or pattern we find also among the various species differences in melanophore number. There is evidence that many of the prospective melanophores which migrate from the crest are not self-differentiating but require some stimulus or material contribution from other cells, notably the ectoderm, in order to produce pigment granules (52, 31, 27, 111). Finally, characteristic differences exist in the morphology of the pigment cells themselves—qualitative differences such as size, type of branching, color or tone, number of granules etc.

By exchanging segments of neural folds containing the crest, in early developmental stages, it is possible to introduce melanoblasts of species exhibiting one characteristic type of pigmentation into individuals exhibiting a different type without changing the topographical arrangement of the tissue environment. Thus it can be determined to what extent the pattern produced by the foreign pigment cells develops according to origin and to what extent it is influenced by the foreign host tissues. When such transplantations are made between embryos of three closely related species of Triturus and their hybrids, the resulting pattern produced by the grafted melanophores in the region of the host into which they are introduced (usually the trunk) is invariably a duplicate of the type found in the donor individual (111, 114). Prospective pigment cells of *T. torosus*, for example, form their normal banded pattern in either *T. similans* or *T. rivularis* hosts; similarly, pigment cells of *T. similans* or *T. rivularis* form their characteristic non-banded patterns in *T. torosus* hosts. The only evidence of any environmental influence on the donor melanophores in these transplantations is seen in their distribution at the border of the yolk mass. In *torosus* hosts, grafted melanophores from either *rivularis* or *similans* fail to descend below the dorsal margin of yolk mass as they would in their normal environments. From these experiments it would appear that the pattern of melanophore distribution is determined primarily by properties intrinsic to the pigment cells.

When such studies were extended to include intergeneric combinations between

either Amblystoma and Triton (99) or Amblystoma and Triturus (114, 115) interesting modification of the basic pattern not observed in the above interspecific transplants became apparent. Under such conditions the pattern produced by the grafted cells does not follow so closely that of the donor type. The paired bands produced by Triton pigment cells in the black axolotl, Amblystoma, for example, are less sharply defined than those of the normal Triton donor; and in the reciprocal combination, the axolotl pigment cells, which in their normal environment become uniformly distributed, show a slight tendency to aggregate in Triton hosts in the regions where Triton bands would normally form. In transplantations from Amblystoma to Triturus the grafted pigment cells show a decided tendency to migrate even beyond the graft region into adjacent host areas where they tend to suppress the development of the host's melanophores, thus bringing about subsequent modifications in melanophore arrangement.

When the exchange of crest is made between donor and host still further apart phylogenetically, such as between anurans and urodeles (3, 4, 64), the pattern formed by the grafted cells bears even less resemblance to that of the donor type. In such cases the final distribution of the grafted melanophores is tremendously influenced by the environment of the foreign host. In all of the aforementioned experiments the morphology of the individual, grafted melanophores is not altered. They retain their specific characteristics as regards size, color intensity, branching etc. and can be readily distinguished from those of the host. The primary change which occurs, therefore, is one affecting their orientation into distinctive patterns.

From the results of transplantations, the xenoplastic type especially, it is evident that factors extrinsic to the melanophores do play an important part in the formation of the definitive pigmentary pattern. What are these factors? Undoubtedly they are many and varied; a few have been somewhat clarified.

By appropriate transplantations between *T. torosus* and *T. torosus-T.similans* hybrids, it has been shown that the factors which condition the peculiar distribution of melanophores at the dorsal margin of the yolk mass are located in the mesoderm of the flank (27). The nature of the 'barrier' is not known. The possibility of its being a purely mechanical block to pigment cell movements has already been mentioned.

It is to be expected that normal organic relationships would be of great importance in pattern formation and this has been shown to be true. In the absence of somites, for example, the melanophores of *Triturus torosus* cannot assume their normal orientation into bands, but remain scattered (111, 113). This clearly indicates that the specific orientation of melanophores, e.g. rearrangement into bands in torosus, is a result of interaction between them and their normal tissue substrate. On this basis it may be possible to explain some of the unusual arrangements occurring when melanophores are grafted to widely unrelated individuals where great differences in substrate must exist. The results of such transplantations indicate that melanophores respond to foreign tissues in a particular way which differs from the response normally given under their own environmental conditions. The response, however, that can be given is in accordance with the specific set of reaction potencies inherent to the melanophore—i.e. provided by its genic composition.

Additional evidence that the rearrangement of melanophores is conditioned by the character of the substrate has been obtained from *in vitro* experiments (113). Outgrowths of *T. torosus* melanophores adhering to the glass cover slip do not undergo rearrangement into dense clusters nearly so readily as those adhering to the inner surface of a hanging drop of medium. Such experiments further show that rearrangements do not take place at all unless the melanophores attain their normal degree of differentiation, i.e. have become fully pigmented. In physiological salt solution *T. torosus* melanophores differentiate slowly and incompletely and remain permanently scattered. In peritoneal fluid, on the other hand, where conditions for complete and normal pigment differentiation are provided, a subsequent rearrangement into dense clusters, never observed in the saline cultures, occurs. This rearrangement appears to be brought about by tensions exerted by the melanophores upon one another by the retraction of the filamentous processes connecting the neighboring cells (112). How do such findings apply to the living organism?

It has been demonstrated that in the normal embryo prospective pigment cells differentiate in a definite sequence (115). Pigmentation begins anteriorly and proceeds gradually posteriorly. This sequence appears to be referable to a corresponding antero-posterior differential in the organic environmental factors influencing melanin pigment formation, since melanophores originating from posterior pieces of crest grafted to anterior levels differentiate at the time appropriate for their new position. Similarly it has been demonstrated that conditions affecting the time of pigment formation are graded along the dorso-ventral axis. If the rearrangement of melanophores is dependent upon a requisite degree of differentiation, as the *in vitro* experiments would seem to indicate, then melanophores situated along the dorsal margins of the myotomes would be the first to fulfill the conditions necessary for rearrangement. Outlying melanophores as they in turn become differentiated would gradually be drawn dorsally towards the region where the tensions, brought about by the retraction of the interconnecting processes, first develop, i.e. along the dorsal margins of the myotomes (113).

In *T. rivularis* the melanophores retain their initial diffuse arrangement throughout larval life. They are inherently incapable of a band-forming response under all of the *in vivo* and *in vitro* conditions so far tested. In view of the results with *T. torosus*, it has been suggested (113) that in *T. rivularis* the development of the pigment cells remains arrested below the level at which the mechanism of reaggregation can be set into play. According to such an interpretation the contrasting behavior of the two species of cells is based on differences which are purely quantitative or relative in nature. Whatever the differences may be, they are clearly referable to factors within the pigment cells themselves, since the melanophores of each species reproduce their characteristic pattern when transplanted to a host of the other species.

The distribution of melanophores is markedly affected by differences in growth rates between donor and host species. Pertinent evidence may be found in examples already cited, where crest is exchanged between Amblystoma and Triturus embryos of the same age. The identical effect, i.e. the spread of donor melanophores beyond the graft region and the subsequent prevention of host melanophores from entering the areas occupied by the grafted melanophores, can be reproduced in embryos having

the same inherent growth rates if crest from older embryos is grafted orthotopically to younger hosts (115). One is led to suspect that at least some of the unusual distribution of pigment cells arising in the anuran-urodele transplantations is related to species differences in growth rate.

The importance of local, environmental factors in the development of the definitive pigmentary pattern in another body region, the eye, has been demonstrated by Barden (5). By combining pigment cells and optic vesicles between species exhibiting marked differences in iris pattern, he was able to show that the resulting pattern followed that of the individual which furnished the structural elements of the eye i.e. the pigment pattern in the iris is controlled by factors located in the iris.

ADULT AMPHIBIAN

The adult amphibian pigmentary pattern is conspicuously different from the larval. In general the adults are more intensely pigmented. Some like *Triturus torosus* darken more or less uniformly and no longer show any indication of the larval stripes. Others like *Amblystoma tigrinum* and *punctatum* develop characteristic light spots on the darkened background. Recently Stearner (107) has studied histologically, in several species of Urodeles, the sequence of changes in the development of the cutaneous pigmentary system, prior to and during metamorphosis, which lead to the formation of the definitive adult pattern. She has been able to identify certain stellate, unpigmented cells in the subdermis and dermis, which occur in close association with the melanophores. The evidence, although somewhat indirect, indicates that some if not all of these cells are potential melanophores. Some differentiate before they migrate into the dermis but the majority enter in the undifferentiated state and later form melanin. The ratio of pigmented to unpigmented cells (obtained from cell counts of a unit area at various developmental stages) is found to be smaller in the advanced larva than it is after metamorphosis is completed. During metamorphosis, the number of melanophores increases rapidly while the number of unpigmented cells shows a general decrease. The developmental changes in pigmentation described by Stearner, both in a normal series and in a series with transplanted neural folds, indicate that a simple relation exists between subdermal, dermal and epidermal melanophores which makes it highly probable that all of these cells arise from one cell type. Accordingly, the only distinction between melanophore types is one based on their position at a given time. Whether this holds for certain anurans where a peculiar type of adepidermal melanophore, functioning as a supporting net work in larval stages, has been described (18, 39) is not clear. Studies on anurans such as those made by Stearner on urodeles are obviously needed to settle this point.

The increase in melanophores at metamorphosis and their appearance in regions where they had hitherto not appeared has been observed in animals with exchanged segments of neural folds (114). For example, transplanted melanoblasts of *Amblystoma punctatum* origin remain undifferentiated, hence produce no pigment in the ventral region of the trunk of *Triturus torosus* hosts until metamorphosis. At this time they differentiate in large numbers and form the typical donor spotted pattern in *T. torosus* hosts which are normally uniformly pigmented. Such results show, without any question, that the melanophores which appear at metamorphosis are

derived from the neural crest and indicate, further, that many are held over in an undifferentiated (unpigmented) state until activated by the metabolic changes accompanying metamorphosis. Although mitoses have been observed in melanophores, it is doubtful whether the phenomenon is common enough to account for the rapid increase observed at this time.

Histological study of the development of the pigmentary system reveals that many important changes take place before the onset of metamorphosis. The development of the dermis, for example, is completed before metamorphosis is begun (107). In some forms (Amblystoma), the striking color change is brought about not only by an increase in the number of dermal melanophores but by their rearrangement within the dermis, just below the epidermis, to form a more complete layer instead of a loose network. Although some melanophores are present in the epidermis they are relatively few in number. The typical yellowish spots result from an aggregation of lipophores in the epidermis and a slight decrease in number of underlying melanophores of the dermis. In other forms (*T. torosus*) the uniform brown pattern, characterizing the metamorphosed animal, is brought about by a large increase in the number of epidermal melanophores which in this species play the major rôle in adult pigmentation. Dermal melanophores are present but they are usually contracted and are masked by the heavy pigmentation of the epidermis.

In *T. torosus*, Stearner observed migration of melanophores from the dermis into the epidermis throughout the larval period, but found it to be particularly prominent at the time when the increase of epidermal melanophores is most rapid. It appears that the morphology of these melanophores is modified by the epidermal environment. Their highly branched processes extend for long distances between the epidermal cells. Most interesting is the observation that the ends of the processes penetrate the epidermal cells, pass over the outer side of the nucleus and form a caplike arrangement of pigment granules. This intimate relation, together with the close correlation between the presence of epidermal melanophores and melanin in epidermal cells, suggests strongly that the melanophores are the source of the melanin pigment granules occurring in the epidermal cells and, thus, strengthens the relationships in this respect between the amphibians and birds and mammals, including man. In view of the observations of Rahn (85) on the deposition of melanin pigment in the epidermal cells of the skin of the rattlesnake by melanophores, reptiles may, also, be added to the list. On the basis of our present knowledge it is quite improbable, in fact, that the epidermal cells of the integument of any vertebrate are inherently capable of autonomous melanin pigment formation.

Birds (Fowl)

The plumage of birds is characterized by a succession of feather types pertaining to definite stages of the life history. In all birds there is a sharp distinction between down feathers and feathers of the definitive plumage (contour feathers). Down feathers are nearly always uniformly colored while definitive feathers may display a wide variety of coloration.

The present status of our knowledge leads to the view that melanin pigmentation

patterns are the result of the interaction of two separate and distinct components: the melanoblasts which produce the pigment granules and the feather germ or papilla which produces the structural elements of the feather. When a feather begins to form, these two components are for the first time brought together. Their relationships in the pigmentation process may be more clearly understood by considering briefly the mechanism of feather formation.

The feather arises from a specialized unit of skin, the papilla, located at the base of the follicle. All feather parts, shaft, barbs and barbules, are derived from the epidermal component of the papilla which becomes specialized to form a ring or collar of embryonic cells encircling the rather massive dermal component. Differentiation of the feather parts progresses from the apical margin of the epidermal collar. The apex of the feather is formed first; successively more basal regions are added by the continuous proliferation of the cells of the collar until the entire feather is formed (see Lillie and Juhn (68, 69), Lillie and Wang (70), Lillie (66, 67) for details). When a feather is shed or plucked the dermal papilla covered by a thin layer of epidermal 'regeneration' cells is left behind. From these cells a new collar forms which gives rise to new feather portions. In contrast to the epidermal component of the papilla which forms anew with each feather regeneration, the dermal papilla remains as a permanent body. If it is removed, the epidermis of the follicle cannot regenerate a feather (70). The dermal papilla functions as a feather 'inductor' and determines the symmetry and orientation of the feather (70). The specific type of feather induced is dependent upon the tract origin of the overlying epidermis (118). A saddle feather papilla, for example, from which the epidermal cells have been removed, transplanted to an empty breast follicle produces a breast feather from the epidermal 'regeneration' cells of the breast follicle and vice versa. Intact breast or saddle papillae, on the other hand, retain their specificity in follicles of either tract (118).

If pigmentation is to take place, prospective melanophores must enter the epidermal primordia of the feather elements. Their entry may be blocked, experimentally, in which case the feather develops normally, but remains unpigmented (90). The most recent evidence indicates that neither the epidermis nor the dermis of the papilla harbors melanoblasts permanently, but each successive generation of feathers acquires a new complement via the dermal papilla from a 'reservoir' of stem cells (melanoblasts) located within the dermis external to the follicle (43). (There is some evidence from recent *in vitro* experiments (76) which would lead us to believe that the dermis exerts an inhibitory influence on the differentiation of melanoblasts into melanophores.) Since pigment cells enter the epidermal collar in an undifferentiated, colorless stage they cannot be detected until pigment granules begin to form. This occurs in a particular region, the zone of differentiation (125) just anterior to the distal portion of the collar where the proliferating epidermal cells are being organized into barb ridges. Above this zone melanophore arrangement becomes definite and orderly. As the cells of the barb ridges gradually attain a certain degree of differentiation, characterized by the onset of cell elongation and keratinization, pigment granules are released from the tips of the melanophore processes or branches into the

cytoplasm of the feather cells (109, 121). Each melanophore deposits pigment granules in only a single small region of the feather during a limited time in development, after which the melanophore ceases to function as a living cell. The completed feather, then, represents a mosaic of enormous numbers of pigment granules laid down by many separate and distinct melanophores.

The respective rôles of the melanophores and the feather germ in the formation of the definitive color pattern have been analyzed by combining melanophores of individuals of species exhibiting one type of color and pattern with feather germs of individuals exhibiting a different color and pattern. Such combinations have been accomplished by means of a variety of methods: 1) By grafting melanoblasts from numerous sources (neural crest, prospective neural crest, skin ectoderm, mesenchyme and from developing barb ridges of regenerating feathers) to host limb buds, prior to the entrance of migratory neural crest cells (30, 35, 126, 87, 88, 80, 110). 2) By grafting prospective limb buds (prior to the entrance of crest cells) to the coelom of pigmented hosts, thus introducing prospective melanophores from the parietal lining into wing skin and feather germs (34, 91). 3) By grafting areas of wing skin and down feathers, experimentally freed of melanoblasts, to the backs of newly hatched host chicks (90). 4) By exchanging areas of back skin between two newly hatched chicks (25, 22). 5) By transplanting feather germs (papillae) between adult individuals (119). Irrespective of the immediate source of the melanoblasts or the method of introducing them into foreign feather germs, the results have been consistent in showing that the melanophores produce their specific color and pattern in homologous feathers of varieties normally exhibiting an entirely different color and pattern. Combinations have been made between all of the common varieties of domestic fowl (including bantams) and other fowl such as pheasants, guinea fowl etc. and even several of the common song birds. In all cases melanophores from varieties with uniformly colored or white plumage (Black or Buff Minorca, Black or White Silkie, etc.) always produce their characteristic uniform color patterns. Melanophores from varicolored varieties (Brown Leghorn, Pheasant, Guinea, Robin, etc.) likewise produce their characteristic varicolored patterns; interestingly enough, melanophores from males and females of varieties showing sex-linked differences in plumage pattern (Barred Plymouth Rock; Rhode Island Red—Barred Plymouth Rock cross) also produce their respective male and female types of pattern in whatever host feather germs they are introduced. The sex genotype of the feather germs in which the melanophores function has no effect on the final pattern produced (127, 128).

Microscopic examination of the barbules of the resulting feathers of the graft-region revealed that the transplanted foreign melanophores deposit in the feather cells melanin granules characteristic of their own particular species (126, 87). It should be pointed out that all of the white varieties of fowl tested (Leghorn, Wyandotte, Plymouth Rock, Silkie) possess melanoblasts which, upon being transplanted to feather germs of pigmented hosts, enter and occupy definite positions in the feather germ where they differentiate into melanophores. However, such melanophores die at a low level in the feather germ before they can deposit pigment in the feather barb or barbule cells; hence the feather is white. The *in vitro* studies of Hamilton (47) have shown that the melanoblasts of the white feathered varieties are all potentially

capable of synthesizing black melanin granules, but are characterized by a lower viability and a higher sensitivity to adverse environmental conditions than those from pigmented varieties. For a discussion of the causal relationships between viability differences and whiteness in feathers see Hamilton (47), Willier and Rawles (126) and Willier (125).

While the large body of data agree in showing that the genotypic constitution of the melanophores is the controlling factor in phenotypic expression of color and pattern, it is also clear that the migration of precursor melanophores into the feather germ and their differentiation and arrangements into definitive patterns are not autonomous but influenced to a great extent by a variety of extrinsic factors. Skin grafting experiments have demonstrated that the invasion of melanoblasts is controlled by the skin and feather germs. Areas of melanoblast-free skin, transplanted soon after hatching to normal host chicks, are readily invaded by melanoblasts from the surrounding host skin (90). But, melanoblasts from the host do not invade a skin graft containing its normal complement of melanoblasts (except occasionally at the margins as evidenced by the presence of mosaic colored feathers, Danforth (22, 23). Such results indicate that some constant ratio between the cells of the skin (and feather germs) must be established and maintained during the process of pigmentation. Foulks (43) has shown that in a normal regenerating feather germ the rate of melanoblast-migration from the dermis differs at different stages during the process of regeneration and, in down feathers, Watterson (121) found that pigmentation is due solely to descendants of melanoblasts which penetrate the feather-forming epidermis during a very limited interval of time in development. It has been established further that conditions within the feather germ determine the orderly arrangement of melanophores, the direction of outgrowth of melanophore-branches and the time of acceptance of pigment granules (Watterson (121) and others).

Other physiological properties of the individual feather germs such as position on the body (tract location), growth rates etc. act as modifying factors and affect the details of the basic type of pattern produced by the melanophores of a particular genotype. Regional differences in feather pattern may be readily observed by examining the plumage of any bi- or multicolored bird. In a normal adult Barred Plymouth Rock, for instance, all the feathers are barred; yet the width and clarity of the bars, relative amounts of black and white etc. vary considerably in feathers from different regions such as wing, tail, breast or back. Each shows its own characteristic type of barring. When melanoblasts from a Barred Plymouth Rock (embryo or adult) are introduced into the developing feather germs of a non-barred individual (Black Minorca, N. H. Red, White Leghorn) these same characteristic differences in the quality of the barring appear in homologous feathers of the graft-region. Similarly, melanoblasts from a non-barred individual, e.g. New Hampshire Red, migrating into feather germs of Barred Rock or White Leghorn origin, produce in saddle feathers the solid red uniform pattern characteristic of the normal New Hampshire Red saddle feathers; in wing feathers the black and red splotched pattern characteristic of homologous New Hampshire Red wing feathers etc. (90).

The black and red response always given by New Hampshire Red melanoblasts in certain wing feathers of different genotypes (Barred Plymouth Rock, White Leg-

horn), in contrast to the all red response in either breast or saddle feathers, is of particular interest as regards the course of differentiation of red and black melanophore types. The presence of these two distinct types of melanophores in the plumage of many other varieties of domestic fowl such as Brown Leghorns, Rhode Island Reds, Silver Campines and various hybrids, and the marked changes in their relative numbers and distribution which may occur with age, hormonal and nutritional changes, have led to much speculation regarding their developmental relationships. Since this topic has been discussed in detail by Willier (124, 125) and Willier and Rawles (127), it need only be mentioned here that the available evidence supports the view that the two types, red and black, differentiate directly from a common precursor cell (melanoblast) rather than the view that red melanophores are merely blacks whose development has been arrested before they reach the black stage. It would appear, then, that melanoblasts of any black-red genotype are potentially capable of differentiating into either black melanophores synthesizing and depositing black, rod-like melanin granules in the feather parts or red melanophores synthesizing and depositing reddish spherical granules: the locus of differentiation, i.e. the epidermal substratum of the particular feather germ or region of the feather germ in which the melanoblast differentiates, determines which of these two potencies is realized. Once a melanoblast becomes fixed (segregated) as to type, black or red, it cannot change to the opposite type. Transitional types have never been observed (24, 48, 81, 110).

It should be emphasized that there is no correlation between the locus of origin of the melanoblasts and their differential response to feather germs of various body regions. Melanoblasts from any region or level—in fact, even melanoblasts from the parietal lining which normally would never enter feather germs—introduced into an area of wing skin, produce in the feathers the color and pattern characteristic of wing feathers (91). The same principle holds for feathers of other regions, breast, saddle etc. Such results lead to the conclusion that in the undifferentiated, melanoblast-stage, pigment cells of any one genotype are all alike.

Rhythmic Patterns—Barring. Not only is there a constant reaction between melanoblasts and feather germ, but also reactions between the individual melanoblasts within the feather germ. The importance of such reactions in the formation of rhythmic, barred patterns has been emphasized by Nickerson (80) on the basis of results from a special study of the Silver Campine and the Barred Plymouth Rock, varieties of fowl exhibiting two genetically and morphologically distinct types of black and white barring. In view of the attempts that have been made to explain the rhythmic pattern formation in barred fowl without considering the melanophores as a distinct part of the pattern-producing system, the recent work of Nickerson deserves more than passing mention.

Nickerson found that melanoblasts from the Silver Campine and Barred Plymouth Rock introduced into feather germs of White Leghorns always produced barred patterns with the barring-period characteristic of the donor. These findings indicated strongly that the periodicity is intrinsic in the melanophores. It was observed further, by appropriate isolation tests, that the prospective white bands or bars of the developing feather contained melanoblasts potentially capable of differentiating and depositing pigment granules in feathers, i.e., the whiteness of the white bands is not

due to an absence of pigment cells, but to their failure to differentiate and deposit pigment granules. In the light of these observations it was suggested that the barring rhythm is controlled through the medium of some diffusible, metabolic substance, produced by the active melanophores in the black band, which inhibits pigment synthesis by the melanoblasts in the subjacent differentiating barb ridges of the prospective white band. As the barb ridges form and increase in length, the source of the inhibitor (black-band) becomes progressively removed from the zone of differentiation with a consequent diminution in concentration of the inhibiting substance. When the concentration falls below the threshold of inhibition, the next series of melanoblasts begins to differentiate and to synthesize and deposit pigment in the feather parts. With the production of pigment, inhibitor substance will again be formed and the cycle repeated until feather differentiation is completed. The above mechanism possesses the characteristics necessary for the production of a time rhythm which must arise as the resultant of the action of two opposed forces: 1) the production of the inhibitor which fluctuates with pigment production and 2) its gradual decrease in concentration with time and with distance from the center of production. Also, the epidermal cylinder of the feather germ, which is devoid of vascular circulation, is an ideal medium for the action of such a mechanism. Strengthening evidence that the production of pigment by the melanophores is an important factor in the control of barring rhythms is found in the fact that the phase of bar formation in a feather germ at any given time is dependent upon the length of time from the beginning of pigment deposition in the feather rather than upon its total age.

In contrast to the Silver Campine, the bar gene of the Barred Plymouth Rock is sex-linked—the female is the hemizygous sex. Since the white bars or bands are wider in the male than in the female pattern of the Barred Plymouth Rock, it would appear that the melanoblasts are inhibited over a greater expanse of developing barbs when the genotype is male than when it is female. This indicates that either the threshold of inhibition is lower in male melanoblasts than in female or that the former produce the inhibitor in greater quantity than the latter. It is to be expected that some such differences in reactivity could show up with differences in genotype (see Willier and Rawles (128) for a discussion of the effects of sex-linked genes on the expression of melanophores in the formation of sexually different pigmentation patterns of feathers of the Barred Plymouth Rock).

As mentioned previously, the details of the barred pattern are modified by many factors involving both the melanophores and the epidermal substratum of the feather germ in which they function. Nickerson tested the validity of his diffusion hypothesis by an analysis of the effects of variations in growth rates, barb ridge size, pigment intensity etc. on the definitive barring pattern. In general, the hypothesis appears to be adequate for explaining the observed differences in barring exhibited by different individuals and by different feathers of the same individual. The nature of the inhibiting substance, however, remains for subsequent work to elucidate. As Nickerson suggests, it may prove to be non-specific, perhaps some substance or substances which act by altering the redox potential and consequently the melanin pigment production of the melanophores (cf. 41).

Mammals (Mouse, Rat, Guinea Pig)

While the development of pigmentation pattern in mammals has not been analyzed nearly as thoroughly as that of birds the experimental results as far as they go are in complete agreement. This is to be expected on the basis of similarities existing in the origin and properties of the melanophores and in the formation and function of the specialized integumentary derivatives, the feathers and the hairs, which in both of these groups are the structures primarily concerned in the development of the definitive pigmentation. It has already been pointed out that prospective melanophores of the mouse arise from the neural crest and are distributed to all parts of the body by extensive migration during early stages in embryonic development. Transplantation experiments have shown that by the twelfth day of gestation these cells are present in all portions of the body (92). Many potential melanophores undoubtedly do not undergo differentiation. Since they cannot be distinguished from accompanying embryonic cells prior to melanin synthesis little is known of their history. In order to participate in hair pigmentation, prospective pigment cells must make their way into the epidermal primordia of the hair elements. In their absence a structurally normal hair can be formed but it cannot become pigmented (89, 92). Hairs like feathers are being constantly shed and replaced throughout life. Each follicle gives rise to a succession of hair generations, each of which requires a new complement of melanophores. So far, little is known of the immediate source of this new complement or how and when during hair regeneration the melanoblasts enter. Presumably they enter via the dermal papilla since it is the only permanent component of the follicle. The pigment cells make their first appearance in the rapidly dividing matrix cells of the hair bulb surrounding the papilla. They deposit pigment granules into the hair cells in a manner similar to that described for the feather and for other epithelial cells (96, 60, 37, 78, 6, 130). After completing their pigmentary function the melanophores degenerate as keratinization of the hair cells sets in. In order to provide pigment for each additional part of the hair as it in turn differentiates, melanophores, obviously, must be continually supplied at the growing base. The completed hair, like the feather, contains numberless melanin granules deposited by many different melanophores functioning at different time intervals during hair development. Thus, any variations or fluctuations in pigment-forming activity of the melanophores will be recorded in the deposition of the pigment granules. It was pointed out by Lillie (67) several years ago that "There is no more convenient or accurate record than the feather once one has learned how to identify the locus of reaction in the germ with the result in the finished feather; in short, to read its autobiography." The same statement holds for the hair. The usefulness of the pigment-granule-record in the finished hair for analyzing the nature of the action of various genes on the pigmentation process in the mouse has been demonstrated recently by Russell (100). In view of the numerous methods that have been employed in the past to analyze pigment-gene action in mammalian skin and hair, this method of approach is of particular interest and promise.

In the mouse as in fowl, melanoblasts, by virtue of their migratory capacity, invade areas of grafted normal skin under proper experimental conditions. Proof has come from homoplastic transplantations of undifferentiated mouse skin. By

removing areas of dorsal and ventral skin from colored and albino donors at birth (or several days earlier) and transplanting them to ventral and dorsal regions, respectively, of new-born hosts of a different color genotype, Reed (93) and Reed and Henderson (94) succeeded in combining pigment-cells of one genotype with hair germs of a different genotype. In all cases, hairs at the periphery of the grafted area of skin, in a zone a millimeter or more in width, developed the color characteristic of the host genotype. (Other hairs of the graft developed the original, donor color showing conclusively that donor melanoblasts were already present in the skin at the time of its removal.) Such results can be explained only on the basis of melanoblast migration. That the host-colored hairs of the graft are not the result of replacement of the grafted epithelium by that of the host is evident from distinct differences in the morphology of hair types developing from dorsal and ventral skin. The hairs of the grafted skin develop always according to origin. These and more recent experiments with other epithelia (13) show that the replacement hypothesis of earlier workers is no longer tenable.

Owing to the fact that many of the genotypes investigated by Reed, and Reed and Henderson displayed color differences in the hair coat of dorsal and ventral body regions, the data (if the writer may interpret them) illustrate further an important point that might otherwise have been overlooked, namely, that the color pattern produced in the grafted hairs by the invading host melanophores is a replica of that found in the hairs of homologous regions of the host. For instance, melanoblasts from the ventral, tan, region of a black-tan host invading a graft of dorsal skin from a colored or an albino donor, produce in the dorsal hairs black, not tan, coloration; similarly melanoblasts from the dorsal, black, region of a black-tan host invading a graft of ventral skin of a different genotype, produce in the ventral hairs tan, not black, coloration. Thus the genotype of the melanoblast provides it with the capacity to develop into a melanophore which synthesizes either black or tan pigment; the epidermal cells of the hair primordia determine which one of the two capacities is realized. The same principle holds for the various other genotypes tested.

As we have already seen in the foregoing section, the identical situation exists in fowl. It will be recalled that host melanoblasts from the saddle region of a New Hampshire Red invading the wing feathers of a wing skin graft of Barred Plymouth Rock origin, placed in the saddle region, produce in the wing feathers the characteristic red-black mottled color pattern of the wing, not the solid red color pattern of the saddle feathers (see page 399).

In hooded rats the migration of melanoblasts into the prospective white hairs developing from a piece of skin from the ventral region of the body grafted autoplastically at birth to a dorsal, prospective black region has been demonstrated by experiments of the writer now in progress. Owing to the fact that hairs developing from dorsal and ventral areas of skin exhibit certain morphological differences (size, texture etc.) and retain these structural characteristics after transplantation to another location, the grafted area is easily distinguishable. Results show that the pigment cells which in dorsal hairs produce a jet-black color pattern, produce in the adjacent grafted hairs of ventral origin a dark-grey color pattern. This latter color is characteristic of the hairs of small ventral spots or flecks occurring now and then in normal

individuals of this strain. So, here again, as in the mouse, we have a demonstration of a variation in pigment-cell response elicited by hair germs originating in a different body region.

In contrast to the results with undifferentiated skin of new-born mice and rats, the hairs regenerating from unpigmented areas of adult (or nearly adult) guinea pig skin transplanted autoplastically to pigmented regions do not become invaded by melanoblasts from the surrounding skin (105, 40, 65). In many cases, in fact, the hair does not regenerate at all (40, 65). The difference in results can no doubt be attributed to the tremendous difference in the developmental age and the physiological condition of the skin at the time of transplantation. Even at birth a guinea pig is much 'older' than a new-born rat or mouse. The work with new-born skin grafts in both the mouse and the rat suggests that melanoblasts from the surrounding skin become incorporated in the grafted follicles at a particular time in development, i.e. during the process of follicular organization. Evidence of a similar nature is available in birds. Watterson (121), for instance, found that melanoblasts enter developing feather germs only during a very limited period of time during the formation of the feather germ. Danforth's beautiful skin transplantation experiments have shown, further, that feathers regenerating from areas of skin exchanged at hatching between two chicks of different color genotypes, retain their original color and pattern indefinitely. (In the chick the organization of the feather papillae is completed approximately 10 days prior to hatching.) At the margins of the graft, however, where certain follicles occasionally undergo reorganization during the process of 'healing in', melanoblasts which have invaded the grafted skin do get a chance to enter and become incorporated in the feather germ, as evidenced by the formation of mosaic feathers of both donor and host color. The evidence, then, points to the view that during the early differentiation of feather or hair papillae some balance or equilibrium between the melanoblasts and the hair- and feather-forming epidermal cells is established and maintained. Following this period of stabilization melanoblast entry is, somehow, blocked.

While the experimental evidence indicates that melanoblasts can enter hair (and feather) papillae only under certain physiological conditions correlated with developmental age, this does not necessarily hold for the more generalized epithelium of the skin which is not concerned with hair formation. In fact, numerous investigators (19, 72, 101, 104, 102, 40, 65, 13, 14) have reported that white skin epithelium from a spotted black and white guinea pig becomes black after it is grafted to a black region; black skin grafted to a white area slowly blackens that of the white area surrounding it. Such experimental evidence indicates that the epidermal epithelium of the skin proper is potentially capable of receiving melanoblasts from an adjacent area after the skin is fully differentiated.

In regard to the development of melanin pigment in the epidermal epithelium of the skin, attention must be called to certain differences that are found normally between mice and rats and guinea pigs which show up, also, in skin grafts. In the mouse and rat the epidermal epithelium does not become pigmented. Except for certain relatively hairless regions, such as ears, nose, tail, soles etc., melanophore differentiation is restricted to the specialized epidermal cells concerned with hair

formation. In the guinea pig, by contrast, the epidermis of the entire body as well as that destined to form hair may become pigmented or, at any rate, is potentially capable of permitting melanophore differentiation. Since most of the workers with guinea pig skin transplants, with the exception of Seevers and Spencer (105), appear to agree that the unpigmented skin epithelium of a white area of the body grafted to a pigmented area elsewhere on the same individual does gradually become pigmented, the question arises as to whether this result is due to the migration of undifferentiated (colorless) melanophores from the surrounding skin or to an 'activation' of those already present in the white region. The principal argument which has been brought against melanoblast migration (79) appears to be based on the assumption that melanoblasts already present in an area of skin prevent the entrance of others. This does not necessarily follow. In the mouse, for instance, we know that melanoblasts are present in the prospective skin of all regions of the body by the twelfth day of gestation. Yet, skin transplanted several days before birth, before any follicular differentiation has begun, and which certainly contains melanoblasts, does receive other melanoblasts from the surrounding skin of the host. Proof has come from transplanting skin between donors and hosts of different color genotypes (homoplastic transplantations) and has already been discussed (pp. 402–403). That host melanoblasts invade the graft is, of course, evidenced by the fact that donor hairs develop the color-pattern of the host genotype. The fact that colored hairs developing in an albino skin graft, for example, are invariably the color of the host and not the masked color of the albino genotype (94) is extremely 'damaging' to any 'activation' conception. The question obviously cannot be settled for the guinea pig by the use of autoplastic grafts in the adult, near-adult or juvenile animal. In view of present-day trends the 'infective cellular transformation' hypothesis of Billingham and Medawar (14, 14a) and Medawar (79) has a certain appeal particularly in explaining their specific results; yet this hypothesis has most certainly not disproved or brought into discredit the known facts regarding melanophore migration clearly established by much careful and painstaking research with amphibians, birds and other mammals.

CONCLUSION

From the data reviewed in this paper we are led to conclude that in the vertebrates, in general, melanin pigmentation in its great variety of forms is produced by one type of highly specialized cell, the melanophore, which originates in the embryonic neural crest. That the melanophores found in all of the vertebrates are homologous cells is evidenced by their origin, by their capacity for extensive migration in their early undifferentiated (melanoblast) stages when indistinguishable from mesenchyme cells, by their general morphology and physiology and, above all, by their capacity to synthesize melanin pigment granules and deposit them in epidermal cells. The data indicate strongly that all melanoblasts of any one individual are initially alike and potentially capable of synthesizing melanin granules. Whether or not this potentiality is realized depends to a great extent upon the particular tissue substrate with which they become associated, in other words, their locus. In the lower, cold-blooded vertebrates, differentiation of melanoblasts into melanophores is, in general, widespread and occurs in a variety of deeper visceral tissues, in the dermis and in the

epidermis. In the higher, warm-blooded vertebrates melanophore differentiation is much more limited and occurs primarily in the epidermis and its specialized derivative, feathers and hair. Associated with the development of these keratinized epidermal structures is the loss of the ability of the melanophore to shift its contents in effecting color changes.

Like any other embryonic cell of the individual, the melanoblast is endowed with a complement of genes and its development and ultimate differentiation are dependent upon the activity of these genes, subject at all times to correlative influences from adjacent or contiguous tissues and to influences from the external environment. While the action of genes in the control of morphological and color patterns in general has not been completely clarified, there is much evidence that genes act by determining the presence or absence of specific enzymes which guide metabolic processes along particular paths or channels (129).

The interpretation, towards which recent investigations on the development of melanin pigmentation in the vertebrates appear to be leading, is that color patterns arise through a constant interaction between melanoblasts and their tissue substrates and to reactions between the melanoblasts themselves. The complement of genes with which a melanoblast of a particular genotype is endowed, provide it with certain reaction potencies which determine the particular type of response that can be elicited by a particular tissue substrate (125). The physiological condition of the substrate may be influenced or 'conditioned' by numerous factors, hormones, vitamins and various stimuli from the external environment, such as temperature, light etc. Changes in the physiological condition of the substrate can elicit changes or modifications in melanophore response, but the specific response is always in accordance with the genic constitution of the melanophore.

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ANTICONVULSANTS[1]

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THE NUMBER AND VARIETY of antiepileptic therapies suggested in the past (200) reflect the frustration of generations of healers confronted by the 'Sacred Disease'. Today, when considerable hope can be extended to the epileptic patient, the armamentarium of clinically acceptable anticonvulsants does not exceed 10 useful preparations. As yet the laboratory methods for screening potentially useful agents are highly empirical, and the probability that any one such agent will receive clinical validation is small.

It is not the purpose of this review to recount the vast literature on clinical effects of the host of drugs which have been tried, in order to rank them in a sort of popularity poll, nor even to cover all the known physiological effects of the most useful agents, seeking a thread of consistency in the tangle of unrelated and desultory observations. At the moment it seems more profitable first to make a frontal attack on the problem of the seizure itself—its mechanism rather than its treatment; then to examine the crucial points in this mechanism which might be advantageously blocked by drugs; and finally to discuss some of the typical actions and structural chemical relations of the clinically useful antiepileptic agents.

MECHANISM OF SEIZURES

That excessive electrical stimulation can produce seizures was demonstrated in the 1870's by Fritsch and Hitzig (62, 103) and by Ferrier (56) in their classical explorations of the excitable cortex of experimental animals. Such studies gave strong confirmation to the speculations of John Hughlings Jackson (106) on the initiation and spread of convulsive activity in the human brain. After more than 70 years, one can hardly improve today upon Jackson's deductions, based on his exhaustive inquiries into aura, onset and pattern of seizures, post-ictal recovery of function and associated interseizure signs of neurological lesions. His primary emphasis was upon the rôle of focal lesions in the various convulsive disorders (pp. 185, 255). The functional aberration of the discharging lesion was well defined by Jackson half a century before the advent of electroencephalography. "Epilepsy is the name for occasional, sudden excessive, rapid and local discharges of grey matter" (p. 100). He inferred that normal brain tissue could participate in the convulsive activity initiated by the abnormal focus, leading to generalization of a seizure (p. 239). He removed the problem of loss of consciousness during petit mal or major seizures from the realm of metaphysics and concentrated instead on the effect of excessive discharge upon the anatomical sub-

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stratum required for more complex nervous function (p. 205). The occurrence of post-seizure depression of function became, in Jackson's hands, a useful anatomical tool and also gave theoretical support to the concept of excessive discharge during a seizure (pp. 145, 183).

Other contemporary advances in the analysis of mechanism of seizures came largely from animal experimentation. Both electrical (7, 60, 141) and chemical methods (64, 125) were used focally for the production of experimental seizures in the latter half of the last century. These experiments raised a new problem. Why, in view of the ease with which seizures could be initiated, did not the activity of neurones in ordinary behavior result in wholesale cerebral explosions? It was necessary to invoke the concept of inhibition. The possible rôle of inhibition in preventing the appearance of seizures was apparently first recognized in 1881 by Bubnoff and Heidenhain (25). They reasoned that stimulation of the gray matter ordinarily produced excitation and inhibition in equal amounts and that the latter restricted the spread and cut short the duration of excitation. According to this concept, seizures occurred either because of excessive stimulation or because, for other reasons, the ratio of excitation to inhibition became greater than unity.

The idea that inhibitory processes must be exceeded by excitatory events to permit the transition from normal behavior to seizures has an inner logic independent of the mechanism of the inhibitory process. A revolutionary conception of the nature of this transition was proposed in 1906 by Sherrington (181). He postulated a transformation of inhibition into excitation under the influence of convulsant drugs or excessive stimulation (p. 112). His examples were drawn not only from spinal cord experiments but also from studies on cerebral cortex (p. 292).

In spite of the most refined modern methods of electrical recording, the mechanism of inhibition has continued to be highly controversial (24, 135, 172). The Brooks-Eccles (24) hypothesis of inhibition contains many implications for the problem of seizures, since it contemplates the possibility that the same short cells which ordinarily inhibit passively can excite actively if they are made to transmit impulses. Thus an anatomical basis is laid for the Gargantuan leap of Sherrington from inhibition to excitation.

The rôle of inhibition in the self-regulation of cortical excitability through reentrant circuits has been vigorously championed by McCulloch (145). The existence of gross inhibitory areas in the cerebral cortex, operating upon cortex through extracortical mechanisms, has been convincingly demonstrated by Dusser de Barenne and McCulloch (42). It would be more difficult to demonstrate a much finer mechanism of inhibition within cortex itself and yet the variety of human experience demands an intracortical mechanism such as that postulated by Bubnoff and Heidenhain and anatomically specified by Brooks and Eccles. Certainly one feature which distinguishes a major seizure from normal behavior is the cataclysmic loss of anything resembling normal reciprocal inhibition even at the lowest levels of nervous integration (96).

The development of methods for the recording of the electrical activity of the human brain has provided convincing evidence for Jackson's surmise that abrupt and excessive discharges are characteristic of seizures and of epileptogenic lesions (18, 71, 108). It is interesting to note that an earlier enthusiasm for classification and inter-

pretation of convulsive disorders on the basis of wave-form in the electroencephalogram (71) has progressively yielded to the Jacksonian emphasis on the value of localization of the discharging lesion (72, 108). Thus psychomotor or psychical seizures have come to be recognized as partial seizures with a focus in either the temporal or frontal lobes (67, 158, 161). Likewise seizures of the petit mal triad have been referred to subcortical midline foci (104, 109, 161) which may be either the seat of a lesion or activated secondarily from other sites.

Not only has electroencephalography confirmed Jackson's concept of occasional sudden, excessive and local discharges, but the more refined electrical studies by Adrian and Moruzzi (2, 155) on single neurones of the pyramidal tract have shown that the seizure discharges are also of high frequency, thus completing Jackson's definition. The importance of these high frequency discharges, elicitable by chemical or excessive electrical stimulation, has been emphasized by Moruzzi, particularly with reference to the manner of invasion of normal brain by excessive activity from a focal lesion.

The anatomical pathways for spread of seizure discharges have been most extensively studied by Rosenblueth *et al.* (175, 176), who used focal stimulation and electrical recording in animals; their work provides an excellent basis for understanding of the march of an aura and other seizure prodromata, as well as for the generally recognized tendency of lesions in some cortical areas to produce convulsive disorders more readily than in others.

Since lesions of the brain do not invariably result in convulsive disorders and because the lesion may be constantly present whereas the seizures are intermittent, the special character of the epileptogenic lesion has received sporadic attention. Certain investigators have stressed instability of the brain as a whole, characterized by sensitivity to changes in such factors as blood gas tensions (68, 71). The Montreal group has placed emphasis on the vascular abnormalities which are demonstrable at the frontiers between normal brain tissue and areas of scarring, etc.; they view seizures as the end-result of progressive neural injury produced by vascular spasm (160). Another point of view is that of Cannon and Haimovici (27), who extended the principle of sensitization by denervation to include the effects of endogenous acetylcholine upon neurones sensitized by loss of afferent connections within the brain. Evidence for this view is not lacking (156, 192).

Most discussions of the nature of seizure foci seem to imply that there is present a collection of pathologically altered neurones which fire excessively when exposed to normal stresses. Another possibility involves the concept of normal neurones exposed to the abnormal stresses of restricted blood supply, etc. and thus forced to discharge excessively. But a third possibility might well be kept in mind. If previous focal injury should have permanently destroyed the smallest (and usually most vulnerable) neurones of a portion of a nerve net, then one might predict from the Brooks-Eccles hypothesis (24) a stable hyperactive region which is deprived of its internal inhibitory mechanisms and therefore capable of responding excessively. This view obviates the necessity for invoking abnormal stress or pathological alteration of function in the individual neurones. When one considers the monotonous regularity of seizures in many patients, continuing for years with no evidence of pro-

gressive neurological damage, one is tempted to consider the possibility of a stable focus of this architectural type.

Penfield and Erickson (159) accept Foerster's characterization of epilepsy as not a disease *sui generis*, but rather as a characteristic response of the central nervous system to a noxious stimulus, which for its development ordinarily requires a predisposing lesion and a precipitating factor. Among the physiological precipitating factors should be considered changes in the *p*H, pO_2, pCO_2, total osmotic pressure and electrolyte composition of the fluid environment of brain cells, as well as changes in body temperature. Endocrine disturbances, nutritional deficiencies and exogenous chemical substances may activate a dormant lesion. Thus a multiplicity of factors may play upon the brain, predisposed by acquired injury or inherited defect, to produce the manifestations of a seizure. Therefore it should not perplex the physician that patients with identical seizure patterns may often respond quite differently to treatment.

In summary and in extrapolation, both clinical and experimental evidence suggests that even small groups of neurones, variously damaged, may give rise to excessively rapid and uncontrolled bursts of discharges. Under certain favorable circumstances these may cause neighboring cells or related centers to discharge excessively. The critical step in the process of secondary involvement may be the appearance of high frequency discharges, the transformation of passive inhibitor cells into excitors, or both. By such a process of self-propagation the entire brain may be set afire in a tonic-clonic seizure, or the conflagration may be retained within bounds to produce manifestations dependent upon its anatomical base. Other centers may then be driven indirectly without themselves participating in the production of seizure discharges. In such a case the centers being driven will not be expected to show post-seizure depression. Perhaps this is the meaning of the characteristic cortical electrical signs and unconsciousness of petit mal, which disappear as abruptly as they began, leaving behind no sign of cortical involvement.

METHODS FOR STUDY OF ANTICONVULSANT DRUG ACTION

The ultimate test of anticonvulsant action must necessarily use patients as test material. The difference in response of different convulsive disorders to a particular medication, well illustrated by the specificity of trimethadione for seizures of petit mal type (128, 131), makes accurate diagnosis an absolute prerequisite for adequate evaluation of the clinical worth of new drugs, particularly in patients with multiple seizure types. Certain complications of clinical trial may tend to discriminate for or against a particular anticonvulsant. For example, the effects of addition of another agent to an established schedule of medication may give an exaggerated impression of the separate worth of the added drug, since synergism may occur, as with trimethadione and diphenylhydantoin (17) or phenobarbital and diphenylhydantoin (32). If the previous medication is too abruptly withdrawn, particularly in the case of phenobarbital, the increased frequency of seizures (113) may be erroneously credited against the new drug (88). An initial exacerbation of seizures, such as may occur with trimethadione (88) or with fasting (132) in cases of petit mal, may prompt the patient or his physician to discontinue medication after an inadequate trial of no

more than a day or two, when perseverance might have resulted in complete control of seizures. The tendency to reserve clinical trial of new agents for refractory patients whose seizures have resisted all ordinary therapy (147) places an undue burden of proof on the test agent.

Since it is highly impracticable and potentially dangerous to test the anticonvulsant action of new drugs directly upon patients without preliminary screening and assay, various laboratory methods have been evolved for such evaluation. For the most part, they have been based on the unquestioned assumption that a clinically effective antiepileptic agent must raise threshold for experimental seizures however induced. Only to a limited extent have methods been varied in order to examine more critically the possible mechanisms of anticonvulsant action.

In general, the methods for initiation of experimental seizures fall into three classes: 1) electrical, 2) chemical and 3) the production of chronic epileptogenic lesions.

1) *Electrical.* For studies of drug action, Albertoni (7) and Bikeles and Zbyszewski (19) used faradic stimulation of the cerebral cortex through the trephined skull; the latter investigators employed relatively prolonged stimulation (30 sec.). Schilf (178) adopted Jellinek's method (110) of stimulation through corneal electrodes and measured seizure threshold for alternating current applied for 0.5 seconds. Spiegel (185) also used corneal electrodes, but measured threshold with both time and intensity as variables, expressing threshold in ampere-seconds. Merritt and Putnam (148) applied rectangular pulses of direct current for 10 seconds through skull and mouth electrodes, as did Knoefel and Lehmann (120). Kozelka and Hine (122) and later Alles *et al.* (8, 9) employed alternating current of fixed strength; shock duration rather than current was varied in their threshold determinations. Bárány and Stein-Jensen (14, 15) passed alternating current through electrodes placed in the external auditory meatus and, in contrast to previous workers, determined the effects of drugs on seizure threshold for both long and short periods of stimulation. In addition, they observed the action of drugs on seizure pattern as well as upon seizure threshold. The reviewers (205) also directed attention to the effect of anticonvulsant agents upon seizure pattern elicited by supramaximal shocks, independently of their effect on threshold, and introduced an assay method based on their observations; they later extended this method to studies on the action of drugs on seizure pattern in non-epileptic human patients undergoing electroshock therapy for psychiatric disorders (204). Hemphill and Walter (99), Kalinowsky and Kennedy (114) and Garciadiego (65) had previously studied the effect of antiepileptic drugs on electroshock seizures in man, but directed their attention to changes in threshold rather than seizure pattern. Swinyard *et al.* (198) introduced the technique of artificially lowering seizure threshold by hydration of experimental animals in order to study the protective action of anticonvulsant drugs.

2) *Chemical.* Albertoni (7) should probably be credited with the first attempt to analyze the efficacy of an anticonvulsant drug (bromide) by means of convulsant agents as well as by electrical methods. According to Moruzzi (155), Landois (125) applied creatine to the cerebral cortex of experimental animals, in the earliest attempt to produce focal seizures by chemical means. Baglioni and Magnini (11) introduced

gressive neurological damage, one is tempted to consider the possibility of a stable focus of this architectural type.

Penfield and Erickson (159) accept Foerster's characterization of epilepsy as not a disease *sui generis*, but rather as a characteristic response of the central nervous system to a noxious stimulus, which for its development ordinarily requires a predisposing lesion and a precipitating factor. Among the physiological precipitating factors should be considered changes in the *p*H, pO_2, pCO_2, total osmotic pressure and electrolyte composition of the fluid environment of brain cells, as well as changes in body temperature. Endocrine disturbances, nutritional deficiencies and exogenous chemical substances may activate a dormant lesion. Thus a multiplicity of factors may play upon the brain, predisposed by acquired injury or inherited defect, to produce the manifestations of a seizure. Therefore it should not perplex the physician that patients with identical seizure patterns may often respond quite differently to treatment.

In summary and in extrapolation, both clinical and experimental evidence suggests that even small groups of neurones, variously damaged, may give rise to excessively rapid and uncontrolled bursts of discharges. Under certain favorable circumstances these may cause neighboring cells or related centers to discharge excessively. The critical step in the process of secondary involvement may be the appearance of high frequency discharges, the transformation of passive inhibitor cells into excitors, or both. By such a process of self-propagation the entire brain may be set afire in a tonic-clonic seizure, or the conflagration may be retained within bounds to produce manifestations dependent upon its anatomical base. Other centers may then be driven indirectly without themselves participating in the production of seizure discharges. In such a case the centers being driven will not be expected to show post-seizure depression. Perhaps this is the meaning of the characteristic cortical electrical signs and unconsciousness of petit mal, which disappear as abruptly as they began, leaving behind no sign of cortical involvement.

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the topical application of strychnine, which had been shown by Magendie in 1819 to produce seizures by its central nervous action. The literature on strychnine action has been reviewed by Dusser de Barenne (41). Recent usage of convulsant drugs in laboratory assay of anticonvulsant potency is examplified in the work of Everett and Richards (50), Pollock and Finkelman (166) and the reviewers (82, 88, 203). The latter have used various subconvulsive EEG phenomena as well as seizures for laboratory anticonvulsant tests. The use of metrazol for activation of EEG dysrhythmia in epileptic patients (116, 216) has also permitted observations of anticonvulsant drug action. Metrazol seizure threshold has been used by Goldstein and Weinberg (77) and Frost (63) to test anticonvulsant drug action in patients.

3) *Chronic Focal Seizures.* Speransky *et al.* (184) introduced the method of focal freezing the cerbral cortex to produce chronic epileptogenic lesions in dogs, a method later adopted by Keith *et al.* (118) Kopeloff *et al.* (121, 157) have produced focal lesions by implantation of alumina cream and other substances. The method has been used only sporadically for observation of anticonvulsant drug action by the latter group of investigators, although it obviously offers a useful method, particularly when combined with EEG studies.

MECHANISMS OF ANTICONVULSANT ACTION

The conceivable mechanisms by which anticonvulsant drugs might prevent, abort, mollify or reduce frequency of clinical seizures should include at least the following broad categories: *a*) action upon the non-neural lesion; *b*) action upon the abnormally altered neurones to prevent their excessive discharge; *c*) action upon normal neurones to prevent their detonation by excessive discharge.

In the first category should be included the possible actions of drugs in reducing the sensitivity of the abnormal vascular supply of an epileptogenic focus. If Penfield's (159, 160) concept of the rôle these vessels occupy in precipitating seizures has even a limited application, then the possibility of anticonvulsant drug effects through vascular action cannot be neglected. This in turn admits the possibility of anticonvulsant effects of a variety of autonomic stimulants and blocking agents, of which few have been studied (199). Aird (3, 4) has interpreted the anticonvulsant action of certain dyes and of desoxycorticosterone acetate as involving stabilization in permeability of cerebral vessels, to add still another possible vascular mechanism.

The second category assumes that, as a result of disease or injury, neurones may exist for a period in a state of hyperactivity, perhaps analogous to that produced by focal application of convulsant substances. It assumes also that drugs which have little effect upon normal neurones may have quantitatively important effects upon those which are pathologically hyperactive. No sharp examples of such a differentiation have yet been presented, although one might point to the ability of diphenylhydantoin to prevent abnormal susceptibility to electroshock seizures in hydrated animals (198), or the general ability of many other anticonvulsant drugs to prevent seizures induced by metrazol, picrotoxin, etc. A pertinent clinical example may be the considerable specificity of trimethadione in eradicating both the spike and wave response and the clinical seizures of petit mal in response to hyperventilation (88, 128, 162). But in general it can be said that agents which modify abnormal excita-

bility or activity of neurones may also be shown to have actions upon normal neurones (202).

Therefore one is led to consider more extensively the third and last category of anticonvulsant action. Clinical experience presents many illustrations of drug action in severing the chain of convulsive activity somewhere between seizure focus and widespread involvement of the normal brain (202). It becomes pertinent therefore to inquire concerning the action of the common anticonvulsant drugs upon properties of normal brain in general and of neurones in particular.

The reported actions of anticonvulsant drugs upon normal brain can be broadly divided into two major groups: *a*) increased threshold for an exciting agent; *b*) decreased responsiveness of the excited system.

The first group has received by far the most attention. The laboratory studies of Merritt and Putnam (148, 168), resulting in the introduction of diphenylhydantoin to clinical practice, have done much to propagate the idea that increased threshold is the *sine qua non* of anticonvulsant action.. The concept is implicit in the procedures of all investigators who have attempted to assay new anticonvulsant drugs and it must be said that almost without exception they have succeeded in demonstrating by various methods that the most commonly accepted anticonvulsant drugs may raise threshold either for electrical or chemical stimulation or both (7–9, 14, 15, 19, 26, 29, 33, 38, 44, 48–51, 63, 65, 70, 75–77, 80–88, 99, 114, 119, 120, 138, 147, 148, 150–152, 164, 166, 168, 169, 173, 174, 185–187, 189, 190, 195–199, 202, 203, 216).

The reviewers (203) have contested this simple concept, particularly with regard to the action of diphenylhydantoin upon electrical threshold for production of minimal or focal seizures, but this does not negate the fact that something akin to an increase in threshold may be demonstrated if only the threshold for more severe seizures is observed.

Less attention has been given to the problem of drug action upon the ability of the brain to respond. Bárány and Stein-Jensen (13, 14) have found that shortening of the tonic phase of experimental seizures is a characteristic action of all the common anticonvulsants. The reviewers (205) have shown that abolition of the extensor component of the tonic phase of seizures produced by supramaximal shocks is a common property of the clinically accepted antiepileptic agents. The possible significance of this phenomenon will be discussed in more detail in relation to the action of diphenylhydantoin (see below).

Bikeles and Zbyszewski (19) postulated that the action of depressant drugs in preventing convulsive responses to faradization of the cerebral cortex was attributable to their ability to reduce temporal summation of repetitive stimuli. Bárány (12), after studying the effects of various anticonvulsants on seizure threshold for shocks of different durations, has found no evidence for such a mechanism.

Bárány (13) discusses another interesting possibility bearing on the problem of anticonvulsant action. He considers that the effect of a small degree of synaptic depression may have little importance in a short reflex chain but that the same effect at each synapse in a reverberating long chain system will be amplified in accordance with the total number of synaptic links in the circuit. Thus small increases in threshold might have little effect upon many normal functions, yet at the same time

make impossible the long-chain reverberation supposedly involved in the spread and maintenance of convulsive activity. An extension of this concept also raises the possibility that small increases in threshold may cause previously excitatory links to become inhibitory, in accordance with the Brooks-Eccles theory of inhibition (24).

One further type of anticonvulsant drug action seems to be sufficiently general to merit description. It has been recently found (202) that peripheral nerve, when subjected to excessive stimulation, may produce double or multiple action potential spikes, somewhat akin to the high frequency discharges described for pyramidal neurones by Adrian and Moruzzi (2, 155). The phenomenon is apparently dependent upon a prolonged depolarization and reduction in threshold, resulting in an extreme degree of supernormal excitability after passage of a single volley of impulses. These effects are prevented by the common anticonvulsants in relatively physiological concentrations. In addition, the repetitive and hypersynchronized discharge of peripheral nerve which is produced by immersion in high concentrations of phosphate is also abolished. Thus direct neuronal actions of anticonvulsant drugs can be demonstrated under conditions of excessive excitability.

An utterly different mechanism of anticonvulsant action must be considered for those drugs, such as amphetamine, which excite rather than depress the central nervous system and which, until the advent of trimethadione, were among the most effective agents in the treatment of petit mal (78, 128, 129, 214). Perhaps glutamic acid (167, 207) should also be considered in this group. When it is recalled that sensory stimulation may occasionally abort cortical seizures (106, 159) and that seizure discharges are much more common in sleep than in the waking state (67), it seems possible that increased activity of normal brain tissue may inhibit discharges from seizure foci. This may be in itself an adequate explanation of the anticonvulsant action of excitant drugs.

CHARACTERISTICS OF SOME COMMON ANTIEPILEPTICS

Bromide. Bromide was the first of the modern antiepileptic drugs. Its introduction by Locock in 1857 (136) for the treatment of catamenial seizures marked a sharp historical break from earlier inadequate methods of treatment (182). Samuel Wilks in 1859 (212) apparently was the first to use bromide (but with iodide) in ordinary epilepsy, thus dissociating the use of bromide therapy from the problem of supposed sexually exciting factors in convulsive disorders. The clinical use of bromide preparations became popular within a short time (89) and introduced an era in which the greatest emphasis was placed on the use of sedative drugs (191). Although progressively superseded by phenobarbital since 1912 and eclipsed by diphenylhydantoin since 1938, bromide continues to find some useful application in the treatment of grand mal seizures in the absence of other seizure types (165, 193). It is less useful in psychic seizures and may exacerbate petit mal attacks (127). The blood levels required to control patients (110–125 mg. %) tend to produce mild sedation and at somewhat higher levels bromide psychosis, neurological disturbances, dermatitis and other signs of bromide intoxication are apparent (79).

Experimental studies of bromide action have been relatively few. Albertoni (7) demonstrated in dogs that bromide antagonized the effects of convulsant agents and

of direct electrical stimulation of the motor cortex. Merritt and Putnam (148), Tainter *et al.* (199) and the reviewers (205) showed that bromide could raise threshold or modify seizure pattern in animals only in doses causing neurological signs. Kalinowsky and Kennedy (114) and the reviewers (204) obtained similar results in human patients undergoing electroshock therapy. Thus the anticonvulsant effect of bromide is relatively poor compared to that of diphenylhydantoin and other agents studied by the above authors. The mechanism of bromide action is unknown. It cannot be solely a matter of replacement of extracellular chloride ion (159) which can be materially reduced in peripheral nerve (139) or in the intact animal (101) without impairment of function.

Phenobarbital. Phenobarbital (5,5-phenyl ethyl barbituric acid) was introduced for clinical trial in 1912 as a sedative and hypnotic on the basis of the studies of Loewe (137), Juliusburger (111), and Impens (105) and in the same year was shown by Hauptmann (97) to be superior to bromide in the treatment of grand mal. Dercum (40) and Grinker (92) were among the first to popularize the use of phenobarbital as an anticonvulsant in the United States. It remained the chief pharmacological weapon against epilepsy until superseded by diphenylhydantoin within the last decade.

The principal limitation upon the use of phenobarbital as an antiepileptic has been its sedative property. Unfortunately the sedative action of bromide and phenobarbital was long considered a requisite for anticonvulsant action, a concept which until recently tended to impede progress in the search for more potent and specific drugs.

Another disadvantage of phenobarbital, but probably not peculiar to this drug alone, is the exacerbation of seizures or even the appearance *de novo* of convulsions after abrupt withdrawal of the barbiturate following a long period of treatment. The phenomenon was commented upon by Hauptmann (97) and has been studied more recently by Kalinowsky (113). Whenever a drug is capable of causing a withdrawal syndrome, one ordinarily expects to find evidence of development of drug tolerance during treatment. Experimentally the occurrence of barbiturate tolerance and cross tolerance has been shown by Green and Koppanyi (90) and Gruber and Keyser (94). Schulz (179) has shown that during phenobarbital treatment there is a progressive decline of plasma cholinesterase, which regenerates more slowly than the rate of disappearance of anticonvulsant action following abrupt withdrawal of phenobarbital. However, this interesting parallelism is not in itself an adequate explanation of phenobarbital tolerance and withdrawal signs and it would be presumptuous to conclude from the work of Schulz that the mechanism of action of phenobarbital is primarily anticholinergic.

The specificity of phenobarbital when compared with other barbiturates has received only occasional attention. Keller and Fulton (119) found that phenobarbital was the only one of several barbiturates examined which could completely abolish the electrical excitability of the motor cortex of monkeys when anesthetic doses were employed. Merritt and Putnam found that phenobarbital in sedative doses was much more effective than other barbiturates or bromide in preventing electrically induced seizures in cats. The reviewers (88, 195, 204, 205) have found

that the outstanding anticonvulsant effect of phenobarbital, as with diphenylhydantoin, is its ability to modifye lectroshock seizure pattern, in which respect it far surpasses various barbiturates having sedative but not clinical antiepileptic action. Taking species differences into account, in general it may be said that phenobarbital is somewhat less effective than diphenylhydantoin in ability to modify seizure pattern. Unlike diphenylhydantoin, phenobarbital possesses definite ability to increase minimal electroshock seizure threshold and to protect animals against metrazol convulsions. In these respects it is in turn inferior to trimethadione. Like diphenylhydantoin but unlike trimethadione, phenobarbital is highly effective in preventing repetitive firing in a supramaximally stimulated peripheral nerve (202).

Mebaral. Mebaral (3-methyl-5,5-phenyl ethyl barbituric acid) is the N-methyl derivative of phenobarbital; it is the barbiturate homolog of mesantoin. Mebaral was first reported by Heyde (100) and Blum (20) in 1932 as an effective antiepileptic in comparison with phenobarbital and in the same year pharmacological studies by Weese (209) suggested a superior margin of safety for mebaral (cf. 177, 204).

Other Barbiturates. Among other barbiturates which have been tried in convulsive disorders, 5-ethyl-5-(1-methyl-1-butenyl) barbiturate (vinbarbital; 'delvinal') has been reported by Davidoff and Doolittle (36) to give favorable results.

Trimethyl barbituric acid and 1-methyl-5-methyl-5-ethyl barbituric acid, particularly the former, have been found effective against grand mal but not petit mal in a preliminary report by Everett (48) who has also demonstrated that these compounds have a high degree of protective action against metrazol seizures in laboratory animals (48, 49, 51). The reviewers (81, 205) found these substances relatively ineffective in modifying seizure pattern.

Pharmacological actions of other barbiturates on the cerebrospinal axis have been extensively studied, but unfortunately for the most part pentobarbital and other sedative barbiturates without strong anticonvulsant potency have been used. The studies are therefore probably more relevant to an understanding of the nature of sedation, sleep and anesthesia than to antiepileptic action. The peripheral nerve studies of Heinbecker and Bartley (98) indicate that while pentobarbital increases threshold it is particularly effective in prolonging recovery time of neurones after impulse propagation. This property has also been demonstrated in thalamic synaptic transmission by Marshall (144), while Jarcho (107) has recently presented evidence that the prolongation of recovery cycle is even more pronounced at the cortical level.

The most direct studies of synaptic depression by barbiturates have been those of Eccles (43) who showed that pentobarbital blocked two-neurone transmission in the spinal cord primarily by increasing the extent of local depolarization required for initiation of propagated impulses by the motoneurone soma; he also presented evidence that the local increase in threshold was related to an increase in membrane resistance. The two-fold action of barbiturates in increasing threshold and prolonging recovery time is probably adequate to account for most of the various types of central depressant actions which have been studied by many investigators (1, 16, 21–23, 39, 59, 73, 126, 210). The central depression caused by most barbiturates may be related in turn to their ability to depress oxidative metabolism of brain in animals and man (47, 55, 91, 171).

Diphenylhydantoin. Diphenylhydantoin (5,5-diphenylhydantoin; dilantin; phenytoin; epanutin) was recommended for clinical trial as an antiepileptic by Putnam and Merritt in 1937 as a result of their careful laboratory studies of the anticonvulsant action of a large number of substances (168). In the following year they reported its superiority over bromide and phenobarbital, thus validating the importance of a rational experimental laboratory search for new antiepileptic drugs (148, 149). Other investigators promptly verified the clinical value of diphenylhydantoin (cf. 147) and it is now generally accepted to be at least the equal of phenobarbital in the therapy of grand mal, as well as an effective agent in the treatment of some cases of psychomotor epilepsy (69, 129, 208), related behavior disorders (61, 170) and symptomatic epilepsies (154).

The successful use of diphenylhydantoin established the important fact that an antiepileptic drug need not be a hypnotic (169). The effects of overdose of diphenylhydantoin both in animals (93, 120) and in man (10, 57, 149) were found to be in general excitatory rather than depressant.

In retrospect, it seems unfortunate that the laboratory methods of Putnam and Merritt (168) were based on the supposed ability of drugs to increase threshold for electroshock convulsions. In the particular case of diphenylhydantoin, the reviewers have been unable to demonstrate any threshold-raising ability in animals when minimal seizures or EEG signs of seizures are taken as the end point (83, 203, 205). Most other investigators (9, 26, 29, 50, 120, 199) have reported increases in threshold of varying degrees, but it is interesting to note that the criteria of convulsive activity taken by these investigators are varied and are much more rigorous than those which one would apply to a patient. Thus the results are complicated by drug-induced changes in seizure pattern. Furthermore the use of long periods of stimulation operates to conceal the occurrence of drug-modified seizures (203, 204). However, when the end point taken is that of minimal seizures, it has been demonstrated that diphenylhydantoin can increase threshold in animals whose excitability has been raised by hydration, although other anticonvulsant agents are much more potent in this respect (198).

There is more general agreement that diphenylhydantoin does not protect animals against convulsant drugs (50, 88, 120). Here again some authors have reported slight protection against metrazol (80, 216) which is more evident with chronic medication (80). But a large number of drugs, including some which could not be classed as useful anticonvulsants, are clearly superior to diphenylhydantoin in ability to protect against metrazol seizures (see fig. 1 for examples).

The same controversy has arisen in connection with the effects of diphenylhydantoin on electroshock seizure threshold in man. Hemphill and Walter (99) and Kalinowsky and Kennedy (114) have reported that diphenylhydantoin increases threshold in patients undergoing electroshock therapy, while the reviewers (204) have interpreted similar observations as representing a change in seizure pattern rather than threshold. Some investigators have reported that pretreatment with diphenylhydantoin necessitates an increase in the quantity of metrazol required to produce seizures (77) or subconvulsive EEG dysrhythmia (216) in epileptic patients, but others have published negative findings (63).

Another problem which has received attention is the effect of diphenylhydantoin

on pattern of seizures. Hemphill and Walter (99) and Kalinowsky and Kennedy (114) observed that atypical seizures were frequent among patients treated with diphenylhydantoin prior to receiving electroshock therapy. Delay and Soulairac (38), Bárány and Stein-Jensen (15) and the reviewers (205) observed that anticonvulsant drugs in general and diphenylhydantoin in particular modified electroshock seizures by reducing or abolishing the tonic phase in animals and in man (204). It was noted that with diphenylhydantoin, in contrast to many other drugs, the modified clonic seizures in animals were quite prolonged (15, 205).

Bárány and Stein-Jensen interpret the change in pattern as representing a greater action of diphenylhydantoin upon subcortical mechanisms responsible for the tonic phase of seizures than upon cerebral cortex (14, 15). Since the medulla in particular has been shown to have a high seizure threshold (95, 96), a selective vulnerability to anticonvulsant drug action might be expected. However, Gley *et al.* (75, 76) have observed that larger doses of diphenylhydantoin are required to modify or prevent seizures in the decerebrate than in the intact animal, while Knoefel and Lehmann (120) failed to find any effect of diphenylhydantoin on seizures in decerebrate preparations. The more rapid post-seizure recovery of animals pretreated with diphenylhydantoin (15, 205) has recently been shown to occur to an equal degree for responses at all levels of integration from medulla to cerebral cortex (202). This finding suggests the possibility that diphenylhydantoin may reduce seizure activity at all levels of the brain, in which case the modification of seizure pattern need not be interpreted on a specific anatomical basis.

Turning now to the effects of diphenylhydantoin upon non-convulsive responses of the brain, the experimental evidence is even less enlightening than in the case of seizures. Gley (cf. 115) found that diphenylhydantoin increased threshold for unsustained motor responses more than for seizures elicited by cortical stimulation. Although the reviewers failed to find an increase in threshold for non-convulsive cortical EEG responses elicited by cortical electrical stimulation, it has recently been demonstrated in their laboratory (202) that thresholds for unsustained movement may be increased particularly when the rate of repetition is slow. No effect of diphenylhydantoin on the recovery process in cerebral cortex was found in these studies.

In an effort to find some neural counterpart of the central actions of diphenylhydantoin, the reviewers (202) have studied various properties of frog nerve. They found that diphenylhydantoin had little effect on various properties including threshold and the recovery process. However, relatively low concentrations (0.04 mmol/l.) prevented the reduction in threshold and the spontaneous firing produced by immersion in isotonic neutral sodium phosphate solutions and also prevented the appearance of repetitive firing in normal nerves subjected to supramaximal shocks. Thus there would seem to be some tangency between the observed effects of diphenylhydantoin upon peripheral nerve and upon brain.

Diphenylene Hydantoin. In addition to diphenylhydantoin, several other hydantoinates tested by Merritt and Putnam (150) were found to possess sufficient experimental anticonvulsant activity to suggest clinical trial (147). For example, 5,5-diphenylene hydantoin showed antiepileptic activity approaching that of di-

phenylhydantoin and similar toxicity except for the appearance of frequent skin rashes and the lack of gingival hyperplasia. This agent had previously been shown to be effective against experimental seizures by Knoefel and Lehmann (120) and also found on preliminary trial to be an effective clinical antiepileptic by Fabing *et. al.* (53).

Mesantoin. Mesantoin (3-methyl-5,5-phenyl ethyl hydantoin) may be considered the hydantoin homolog of the barbiturate mebaral. Initial clinical trial by Loscalzo (140), Clein (30), Kozol (124), Lennox (130) and Marburg and Helfand (143) showed that mesantoin could control many grand mal and psychomotor cases which were refractory to diphenylhydantoin or other medication. Only the last-named authors reported favorable results in petit mal. The toxicity of mesantoin differs from that of diphenylhydantoin in that sedation and skin rashes occur more frequently, while gingival hyperplasia has not been reported. Thus combinations of mesantoin and diphenylhydantoin can be employed for summation of their therapeutic properties with lessened likelihood of toxicity (124).

The clinical use of mesantoin was suggested by the studies of Tainter *et al.* (199), who found that it was highly effective in raising electroshock threshold in experimental animals. It was found more effective than diphenylhydantoin in altering seizure pattern in experimental animals (197), and resembled diphenylhydantoin in similar tests with human electroshock seizures (204). The reviewers have also shown that it differs from diphenylhydantoin in exhibiting some protective action against metrazol seizures, a property apparently related to N-methylation in several heterocyclic anticonvulsants (fig. 1).

Phenyl Thienyl Hydantoin. The thienyl derivative of diphenylhydantoin (5-phenyl-5-thienyl hydantoin) differs from diphenylhydantoin only in the substitution of a more unstable thienyl ring for one of the 5-phenyl groups. Peterman (163) has considered this compound to be superior to diphenylhydantoin in grand mal and petit mal, but clinical studies are not yet adequate for comparison. The reviewers have noted that it may be successfully substituted for diphenylhydantoin when gingival hyperplasia limits the tolerated dosage of the latter. Their laboratory studies indicate that there are no important differences between the two drugs when compared by various anticonvulsant tests (85).

Trimethadione (*'Tridione'*). The specificity of trimethadione ('tridione'; 3,5,5-trimethyl-oxazolidine-2,4-dione) in the treatment of petit mal seizures was briefly reported by Perlstein in 1945 (162, 174) and confirmed in the extensive studies of Lennox (128, 131, 133) and within a short time by many other clinical investigators. It was also reported to be useful in at least some cases of grand mal (201), psychomotor seizures (37) and cerebral palsies (35, 162) and was shown to have clinical analgesic properties (173). Its usefulness in major seizures has been seriously questioned by Lennox (131), but the reviewers have convinced themselves of its general antiepileptic value when it is combined with diphenylhydantoin in cases which yield to neither drug alone (17); dramatic results may frequently be obtained in cases of grand mal particularly when associated with petit mal EEG (88, 202). Even petit mal seizures are occasionally exacerbated during the first few days of therapy in cases which ultimately respond favorably to trimethadione (88).

on pattern of seizures. Hemphill and Walter (99) and Kalinowsky and Kennedy (114) observed that atypical seizures were frequent among patients treated with diphenylhydantoin prior to receiving electroshock therapy. Delay and Soulairac (38), Bárány and Stein-Jensen (15) and the reviewers (205) observed that anticonvulsant drugs in general and diphenylhydantoin in particular modified electroshock seizures by reducing or abolishing the tonic phase in animals and in man (204). It was noted that with diphenylhydantoin, in contrast to many other drugs, the modified clonic seizures in animals were quite prolonged (15, 205).

Bárány and Stein-Jensen interpret the change in pattern as representing a greater action of diphenylhydantoin upon subcortical mechanisms responsible for the tonic phase of seizures than upon cerebral cortex (14, 15). Since the medulla in particular has been shown to have a high seizure threshold (95, 96), a selective vulnerability to anticonvulsant drug action might be expected. However, Gley *et al.* (75, 76) have observed that larger doses of diphenylhydantoin are required to modify or prevent seizures in the decerebrate than in the intact animal, while Knoefel and Lehmann (120) failed to find any effect of diphenylhydantoin on seizures in decerebrate preparations. The more rapid post-seizure recovery of animals pretreated with diphenylhydantoin (15, 205) has recently been shown to occur to an equal degree for responses at all levels of integration from medulla to cerebral cortex (202). This finding suggests the possibility that diphenylhydantoin may reduce seizure activity at all levels of the brain, in which case the modification of seizure pattern need not be interpreted on a specific anatomical basis.

Turning now to the effects of diphenylhydantoin upon non-convulsive responses of the brain, the experimental evidence is even less enlightening than in the case of seizures. Gley (cf. 115) found that diphenylhydantoin increased threshold for unsustained motor responses more than for seizures elicited by cortical stimulation. Although the reviewers failed to find an increase in threshold for non-convulsive cortical EEG responses elicited by cortical electrical stimulation, it has recently been demonstrated in their laboratory (202) that thresholds for unsustained movement may be increased particularly when the rate of repetition is slow. No effect of diphenylhydantoin on the recovery process in cerebral cortex was found in these studies.

In an effort to find some neural counterpart of the central actions of diphenylhydantoin, the reviewers (202) have studied various properties of frog nerve. They found that diphenylhydantoin had little effect on various properties including threshold and the recovery process. However, relatively low concentrations (0.04 mmol/l.) prevented the reduction in threshold and the spontaneous firing produced by immersion in isotonic neutral sodium phosphate solutions and also prevented the appearance of repetitive firing in normal nerves subjected to supramaximal shocks. Thus there would seem to be some tangency between the observed effects of diphenylhydantoin upon peripheral nerve and upon brain.

Diphenylene Hydantoin. In addition to diphenylhydantoin, several other hydantoinates tested by Merritt and Putnam (150) were found to possess sufficient experimental anticonvulsant activity to suggest clinical trial (147). For example, 5,5-diphenylene hydantoin showed antiepileptic activity approaching that of di-

phenylhydantoin and similar toxicity except for the appearance of frequent skin rashes and the lack of gingival hyperplasia. This agent had previously been shown to be effective against experimental seizures by Knoefel and Lehmann (120) and also found on preliminary trial to be an effective clinical antiepileptic by Fabing *et. al.* (53).

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Toxic signs have been reviewed by Lennox (131). This side-effect most frequently reported by all investigators is hemeralopia (photophobia), an unusual form of toxicity which seems to depend upon the retinal rather than central action of trimethadione, according to Sloan and Gilger (183). As with many other drugs, frequent skin rashes and occasional blood dyscrasias are observed; reversible renal damage has also been reported. In large doses the drug has a sedative action (162).

Trimethadione was synthesized and first reported to have analgesic properties by Spielman (188). Other oxazolidine-2, 4-diones had previously been shown by Erlenmeyer (46) to have central nervous depressant actions and in particular propazone (5,5-di-*n*-propyloxazolidine-2, 4-dione) had been tried in animals and man as a hypnotic and anticonvulsant by Luton *et al.* (142) and Stoughton and Baxter (194). Everett and Richards in 1944 (50) reported that trimethadione raised thresholds both for chemically and electrically induced seizures. The reviewers (81, 86, 88) extended these laboratory observations using a number of assay methods and concluded that the outstanding property of trimethadione was its protective effect against metrazol-induced seizures and subconvulsive phenomena. Although as effective as phenobarbital in raising electrical seizure thresholds, it was found far inferior to phenobarbital and diphenylhydantoin in ability to modify maximal seizure pattern in man (204) as well as in animals (205). This latter feature would seem to correlate with its relative ineffectiveness in grand mal. In acute spinal animals it was found to depress multineuronal more than two-neuronal reflexes (88), an action possibly correlated with its known clinical analgesic properties (173) In peripheral nerve studies (202) it was found much inferior to the usual anticonvulsants in ability to prevent repetitive firing.

It will be seen that trimethadione differs from the anticonvulsants previously discussed in clinical specificity and toxicity and in laboratory tests. But as yet there seems to be no adequate laboratory test for distinguishing drugs which would be effective against petit mal, although attempts to reproduce the laboratory counterpart of petit mal have not been lacking (28, 88, 109).

Paradione. Paradione (3,5-dimethyl-5-ethyl oxazolidine-2,4-dione) was reported by Everett (48) to have high antimetrazol potency and to possess clinical effectiveness against petit mal. The studies of Lennox and Davis (133) indicate that there is little clinical difference between paradione and tridione except perhaps for a somewhat less frequent occurrence of hemeralopia with the former. From the investigations of Spielman and Everett (190) on structure-activity relations of trimethadione congeners, it seems improbable that longer alkyl substituents will bestow greater clinical effectiveness against petit mal.

Epidon. Epidon (5,5-diphenyl oxazolidine-2,4-dione) has been reported by Ellermann (44, 45) to be clinically effective in grand mal but not in petit mal and to have anticonvulsant properties comparable to those of diphenylhydantoin in laboratory studies. As may be seen in figure 1, epidon is superior to trimethadione and paradione in ability to modify seizure pattern but is relatively ineffective against metrazol. Pfeiffer (164) has recently observed that 5-phenyl oxazolidine-2,4-dione is superior to the diphenyl congener (epidon) and a number of others in raising metrazol seizure threshold and in modifying electroshock seizure pattern. Epidon

has been found comparable with diphenylhydantoin rather than with trimethadione in peripheral nerve studies (202).

Phenacetylurea (Phenurone). Phenacetylurea ('phenurone') has recently been been reported effective in selected refractory cases of grand mal, petit mal and psychomotor seizures by Gibbs, Everett and Richards (70). Observed toxic signs were few, with anorexia, cachexia, headache, insomnia, palpitation and questionable drug rash occurring in diminishing order of frequency. A most interesting result of phenurone therapy was an exacerbation of preexisting personality disorders in patients with psychomotor seizures. The drug is one of a large series of conceivable degradation products of anticonvulsant substances. It was synthesized by Spielman (189) and is essentially 5-phenyl hydantoin with an opening of the hydantoin ring between the 1 and 5 positions. It was found by Everett (49) to be one of the most effective agents yet tested against chemically and electrically induced seizures in laboratory animals. The heuristic implications of the as yet limited experimental and clinical results obtained with phenurone are many.

Glutamic Acid. Price *et al.* (167) reported that glutamic acid was effective in some cases of grand mal and psychomotor seizures and subsequently the *l*+ form was shown by Waelsch and Price (207) to be the active isomer. Other central nervous actions were also reported, including enhanced maze learning ability in rats (215) and improved behavior and performance in subjects with mental deficiency (5). That any favorable action should be found with small oral increments of the most common dietary amino acid seems surprising. Laboratory studies by the reviewers (82) have failed to show any anticonvulsant action of glutamic acid in various species of experimental animals.

Steroids. McQuarrie, Anderson and Ziegler (146) have reported a reduction in frequency of grand mal seizures in patients treated with desoxycorticosterone acetate, but their results were not confirmed by Aird (4). Aird was able to detect a protective action of the steroid against procaine convulsions, as had Selye (180) against metrazol seizures; but, in contrast to Woodbury and Davenport (213), he was unable to confirm Spiegel's (186, 187) demonstration of the ability of desoxycorticosterone to raise seizure threshold. Since Spiegel has found that several steroids without common physiological effects have laboratory anticonvulsant potency, and in view of the paucity of clinical data, the significance of the desoxycorticosterone studies cannot yet be assessed.

Carbon Dioxide. The ease with which seizures of the petit mal group may be precipitated by hyperventilation and suppressed by CO_2 inhalation (71) places CO_2 in the category of a physiological anticonvulsant. Cerebral blood flow may be increased by CO_2 excess (74), but a direct neural effect should also be considered in the light of observations that carbon dioxide may increase threshold in peripheral nerve and prevent repetitive firing induced by a wide variety of stimulating substances (139).

Miscellaneous Antiepileptic Therapies: Fasting; Ketogenic Diet; Dehydration. The use of dietary measures is among the most ancient of all therapeutic attempts to control the 'Sacred Disease' (200). Simple fasting was one of the more prominent of these measures and has even had a desultory trial in the present century. It is

interesting to note in some seizure charts published by Lennox and Cobb (132) that with fasting there may be an initial exacerbation of seizures particularly of the petit mal group, even in patients who subsequently show complete remission of attacks with continued rigorous fasting. However, starvation is at best a barbaric practice in the eyes of most patients. Geyelin (66), who reintroduced the method in the modern period, suggested that the favorable effects were due to acidosis. Wilder (211) proposed and successfully used a high fat diet in an effort to produce acidosis. Keith (117) has recently reevaluated the efficacy of the ketogenic diet after a trial of several decades in a large series of patients and concluded that it compares favorably with the use of anticonvulsant drugs.

The possibility that the success of the ketogenic diet was attributable to products of intermediate fat metabolism was considered by Wilder, who suggested acetoacetic acid as a possible physiological anticonvulsant. Recently the glycerol moiety has been tested by Kajdi and Livingston (112) with results equal to those of the ketogenic diet itself.

In general, the literature suggests that dietary measures are more useful in convulsive disorders of the petit mal group. Therefore it is not surprising that laboratory studies fail to reveal obvious anticonvulsant effects of dietary changes. In fact, Davenport and Davenport (34) have found an increased susceptibility to electrical and chemical seizures in rats which were starved as well as in those given a high fat diet.

Rigorous fluid restriction was introduced by Fay (54) in 1929 for the therapy of convulsive disorders, concomitant with the introduction by McQuarrie (see 146) of a pitressin-hydration test for precipitating seizures as a diagnostic procedure. The reviewers (203), in evaluating previous studies upon the relation between water balance and electroshock seizure threshold (198), have concluded that total extracellular electrolyte concentration is an important determinant of seizure threshold. An excess of sodium ion, the predominant ion of extracellular fluid, has recently been shown by Woodbury and Davenport (213) to increase seizure threshold in laboratory animals.

STRUCTURE-ACTIVITY RELATIONS

The search for more effective anticonvulsants passed from the era of trial and error with the publication by Merritt and Putnam of their extensive studies on hydantoinates and other related chemical nuclei, culminating in the clinical trial and validation of diphenylhydantoin (148, 168, 169). Although other investigators have studied the effects of modification of molecular structure on anticonvulsant activity (8, 9, 26, 29, 33, 48, 51, 87, 120, 164, 187, 189, 190, 195, 197, 199, 205), the most extensive contribution has continued to come from Merritt and Putnam and their associates (138, 147, 150–152).

Before attempting to analyze the structural relationships necessary for anticonvulsant activity, it might be well to set down certain general principles for orientation:

(a) *The ultimate value of an anticonvulsant drug must be determined in clinical practice.* Only to the extent that laboratory methods of assay correlate well with the

clinically established potency of a series of drugs can the laboratory methods be validated. Even then it is not always possible to forsee prohibitive toxic side-actions which are peculiar to the human species.

(b) *The convulsive disorders differ radically in their responses to therapy.* To cite but one example the specificity of trimethadione for seizures of the petit mal triad and the lack of effect of diphenylhydantoin in most such cases (128) illustrate the futility of a search to find a single agent for the treatment of 'epilepsy' in the singular, or of reliance upon a single laboratory test to determine the potential anticonvulsant activities of a given substance.

(c) *There are many possible mechanisms of anticonvulsant action.* Some of these mechanisms have already been discussed and it is obvious that each of them requires a different type of laboratory analysis for its detection. Therefore, as emphasized also in the preceding paragraph, there are cogent reasons for using a battery of laboratory tests.

(d) *The measure of usefulness of an agent is not its absolute potency but its therapeutic index.* The comparison of drugs on a weight or molar basis for their laboratory anticonvulsant potency may have a limited theoretical value, but a more important consideration, especially to the patient, is the ratio between the dosage of a particular drug which causes untoward effects and that which adequately controls seizures. The higher the ratio, the more useful the drug, regardless of the absolute dose levels.

The reviewers have attempted to carry out these principles by developing a battery of tests and expressing the specificity of each drug as a 'protective index' (84, 87, 203). Table 1 illustrates the contrast between two such anticonvulsant tests when used for analysis of a series of drugs including both clinically effective and ineffective agents. The reviewers limit themselves to data from their laboratory (87, 195, 196, 197, 205), since the literature does not provide adequate information for cross comparison of indices. The table gives only a few representative examples in the three most widely-studied heterocylic groups. The tests are roughly comparable to those of Pfeiffer (164) and Spielmann (189). The supramaximal test may also be cautiously compared with electroshock threshold tests of the many other investigators who have used severe seizures as end points. Accepted clinical uses of the drugs are indicated where possible. The structural modifications are also given, starting from the basic configuration shown in figure 1.

It should be emphasized that the nuclei represented in table 1 are not the only structures having anticonvulsant activity (cf. 150). However, members of these three groups together with their degradation products (189) comprise most of the clinically useful antiepileptic drugs. It should also be emphasized that experimental data derived from the various types of electroshock seizure tests are extensive, while metrazol studies are relatively meager. With these reservations in mind, it is possible to draw several tentative conclusions from the literature and to illustrate a few of them with the findings given in table 1.

(a) A 5-phenyl group is important but not critical for antielectroshock activity. It may be replaced by a naphthyl ring (138, 150) but not by cyclohexyl, benzyl or various other rings or by substituted or unsubstituted alkyl chains without considerable loss in activity (8, 9, 150, 164, 169, 189). For example, a comparison of the

TABLE 1. COMPARISON OF CHEMICAL STRUCTURE, LABORATORY ASSAY AND CLINICAL USEFULNESS

NUCLEUS AND DRUG	SUBSTITUENTS[1]			CLINICALLY USEFUL IN:[2]	EXPERIMENTAL PROTECTIVE INDEX	
	R_1	R_2	R_3		Supramaximal electroshock[3]	Metrazol[4]
Hydantoins:						
Mesantoin	C_6H_5	C_2H_5	CH_3	GM, PsM	11.1	0.9
5,5-phenyl thienyl hydantoin	C_6H_5	C_4H_3S	H	GM, PsM	4.5	0
Nirvanol	C_6H_5	C_2H_5	H		3.4	0.6
Diphenylhydantoin	C_6H_5	C_6H_5	H	GM, PsM	2.4	0
3-methyl diphenylhydantoin	C_6H_5	C_6H_5	CH_3		0.9	0.6
Barbiturates:						
Mebaral	C_6H_5	C_2H_5	CH_3	GM	2.5	2.5
Phenobarbital	C_6H_5	C_2H_5	H	GM	3.6	0.9
Diphenyl barbituric acid	C_6H_5	C_6H_5	H		1.8	0.4
Trimethyl barbituric acid	CH_3	CH_3	CH_3	GM	1.0	1.5
Barbital	C_2H_5	C_2H_5	H		0.7	1.1
Oxazolidine-2,4-diones:						
Epidon	C_6H_5	C_6H_5	H	GM	1.8	0
Trimethadione	CH_3	CH_3	CH_3	PM	1.2	1.5
Propazone	C_3H_7	C_3H_7	H		1.0	0
Paradione	C_2H_5	CH_3	CH_3	PM	0.7	1.2

[1] See fig. 1.

[2] *GM:* Grand mal. *PsM:* Psychomotor seizures. *PM:* Petit mal.

[3] Supramaximal index determined by dividing dosage of drug causing minimal neurological signs in 50% of rats by dosage abolishing extensor tonic component of seizure in rats subjected to a shock of five times threshold strength (150 mA alternating current delivered for 0.2 sec. through corneal electrodes).

[4] Metrazol index determined by dividing minimal neurological dose as above by dose preventing seizures in 50% of rats after subcutaneous injection of metrazol (70 mg./kg.).

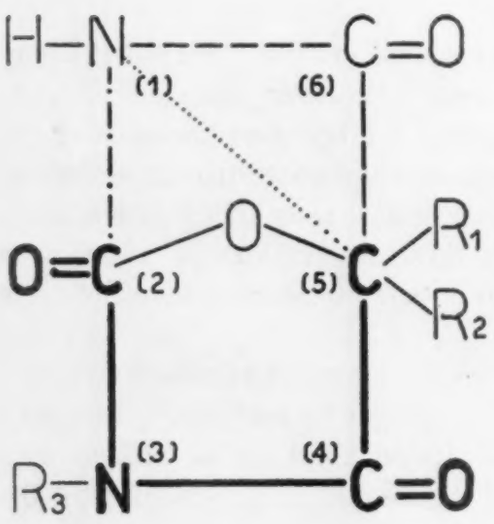

Fig. 1. STRUCTURAL RELATIONSHIPS of clinically available antiepileptics. *Bold face:* Common denominator. *Dash line:* Barbiturate nucleus. *Dotted line:* Hydantoin nucleus. *Thin solid line:* Oxazolidine-2,4-dione nucleus. Opening of the hydantoin nucleus between positions (1) and (5) yields the corresponding acetylurea.

supramaximal electroshock indices of phenobarbital and barbital in table 1 shows the clear superiority of the former.

(b) The addition of a second 5-phenyl group reduces antielectroshock activity in comparison with the effect of a short 5-alkyl chain, as may be seen by contrasting nirvanol with diphenylhydantoin or phenobarbital with diphenylbarbituric acid in table 1. Similar results for these and other pairs are to be found in the literature (9, 150, 164, 169, 189). Optimum activity among the 5-phenyl-5-alkyl compounds seem to occur with short alkyl groups (9, 138). Alkyl substitutions are more effective than their alkoxy- or alkylthio-homologs (26, 152).

(c) A reactive group at position 4, and particularly a carbonyl group, increases antielectroshock activity (150, 169).

(d) N-methylation at position 3 has been shown to reduce antielectroshock potency and specificity at least among the barbiturates (196). Although this effect may be seen by comparing mebaral with phenobarbital in table 1, the opposite effect is illustrated by two hydantoinates, nirvanol and mesantoin. N-methylation seems to improve the antimetrazol potency of all three groups of compounds in table 1. Indeed this is the only tentative correlation that can be made between chemical structure and metrazol index in this series.

(e) A reactive group at position 2 may not be essential (168), but a 2-keto group contributes to high activity (150) and is superior to the homologous 2-thio derivative (26) in electroshock tests.

(f) Beyond position 2, the optimal structure is yet to be determined. In addition to various heterocyclic rings, several straight-chain compounds and particularly the phenacetylureas possess high indices in metrazol as well as in electroshock tests (189). Although it is conceivable that the heterocyclic rings are opened *in vivo* to yield a common anticonvulsant substance (169), available information on the metabolism of anticonvulsants (cf. 102, 103) is too sparse to justify this unitarian concept.

From the above considerations, it can be said that 5-phenyl-5-alkyl compounds having the common denominator shown in figure 1 possess superior ability to modify electroshock seizure pattern and to raise threshold for generalized seizures. Since the indices obtained by various investigators using these tests have correlated to a fair degree with clinical usefulness in the treatment of grand mal, continued study of this group of substances is warranted by the clinical need for new effective antiepileptics of low toxicity.

No such general conclusions can yet be drawn concerning the relation between chemical structure, laboratory assay methods, and effectiveness against convulsive disorders of the psychomotor or petit mal types. The apparent specificity of hydantoinates for psychomotor seizures and of oxazolidine-2,4-diones for petit mal, suggested by table 1, is belied by the reported effectiveness of phenacetylurea in both of these disorders (70). If further progress is to be achieved other than by trial and error in the treatment of these seizure types, it becomes most urgent to study their basic pathology more intensively, and if possible to reproduce them experimentally in laboratory animals.

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GASTRIC ABSORPTION

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ALTHOUGH EXPERIMENTAL EVIDENCE regarding gastric absorption was presented as early as 1808 (71, cf. 70), the general impression prevails that relatively little work has been done on the problem of stomach permeability and that only few substances are absorbed from the gastric cavity in amounts which might lead to the conception of the stomach as an absorptive organ (8, 9, 67, 101). Yet, since the gastric mucosa and the stomach walls are permeable to ethyl alcohol, for example (53), it becomes apparent that gastric absorption may be of some physiological significance in nutrition and therapeutics and of special significance in toxicology and allergy, despite the fact that the capacity of the stomach for absorption is not nearly so great as that of the intestine.

No review of what has been accomplished in the studies of absorption from the stomach would be complete without mention of the procedures used, particularly since divergent conclusions regarding absorption are often the consequence of differences in methods.

Among the experimental techniques in determining gastric permeability, closure of the pyloric region in order to obviate contact between the material investigated and the highly absorptive small intestine has been one of the most frequently used (7, 10, 16, 17, 29, 38, 68, 69, 78, 79, 97, 99, 104, 109), the substances being introduced into the stomach by injection, by ingestion, by stomach tube or by fistula (2, 57, 60–66, 74, 86, 93, 105, 108, 111, 112). Even more frequently employed in investigations has been ligation of both the cardiac and pyloric regions of the stomach, the test material being introduced by means of a small-bore hypodermic needle (4, 33–35, 39, 40, 42, 50, 53, 54, 56, 71, 75, 76, 77, 98, 107, 114).

Of these two methods, the latter is preferable, since in its use one does not have to be concerned with the possibility of absorption by the esophagus, as the test material is passing along it to the stomach, or with the problem of regurgitation. On the other hand, ligation of cardia and pylorus introduces the possibility of creating abnormally high 'filtration' or hydrostatic pressures in those instances where appreciable volumes of fluid may be injected (cf. 26, 27, 53). However, where ligation techniques are to be employed, cannulation at either the cardiac or pyloric end of the stomach is an obvious way of solving the potential problems of either esophageal absorption or increased hydrostatic pressure.

Aside from the objections mentioned, there are two others: one pertains to the condition of the stomach following ligation; the other, to the element of time. While it is true that the ligated or the ligated and cannulated stomach or the stomach with fistula is unphysiological, it will be conceded that abnormal conditions must of necessity be the case in many instances of biological experimentation and that, where comparative data are obtained under identical conditions, the validity of the comparisons is not impaired. More serious, however, is the criticism that in attempts to demonstrate gastric permeability, many investigators have allowed the substance tested to remain in contact with the gastric mucosa for inordinately long periods, even considering the length of time necessary for complete emptying of such materials as fats, proteins and carbohydrates.

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Still another technique employs the principle of blocking the opening between stomach and duodenum by use of an inflatable balloon (12, 93, 111, 113). While this method has the advantage of being more physiological than the preceding ones, reproducibility of closure is more exact in the former, in which, also, leakage is less likely to occur.

Some investigators have closed the pylorus by introducing acid into the duodenum just below the pylorus, the pylorospasm thus produced being said to last for several hours (22, 94). Here, too, the questions of leakage and of exact reproducibility of extent of closure from animal to animal have yet to be satisfactorily answered.

The Pavlov and Heidenhain pouch methods have also been frequently employed (18, 19, 30–32, 55, 59, 72, 89). Although the problems inherent in the previously mentioned methods are not encountered here, absorption studies by these procedures have been usually confined to only a part of the stomach; whereas, frequently, conclusions have been referred to the entire stomach.

The method of introducing two substances simultaneously, the gastric absorption characteristics of one being known, has been used occasionally, although this procedure is not popular. In the latter method, the pyloric opening is left unligated. After a given time interval, the stomach contents are withdrawn and the amount of 'unknown' absorbed is determined and expressed as a percentage based on the amount of the 'known' present and the amount previously determined as being absorbed under the experimental conditions (49, 51, 83). This method fails to account for the possibility of differential expulsion into the intestine.

Finally, in studies on the absorption of inorganic iron, fats and dyes, a few investigators have employed the histological technique, examination being made of the gastric mucosa (1, 41, 44, 48, 78, 92).

WATER

The gastric absorption of water has been studied in human beings, dogs, cats, sheep and calves. Of the various investigators involved in these studies, Bönninger (10), Edkins (26), Jaworski (51) and von Mering (112) presented data which purported to show that absorption of water does not occur or occurs only to a negligible extent, while the results of Brandl (12), Feitelberg (30), Klimov and Kudriavtsev (55), Pfeiffer (85) and Trautmann (108) suggested the contrary.

Little credence, however, should be given to the results of the former group, since either the methods employed were faulty or the conclusions did not logically follow the experimental data. In the latter group, too, criticism may justifiably be leveled at the findings of Brandl (12), because of his use of the balloon technique, and especially at those of Pfeiffer (85). The evidence presented by the others in the second group, particularly that appearing in the report of Feitelberg (30), is, however, convincing.

Finally, in 1943, Cope, Blatt and Ball (18) investigated the exchange of water across the gastric membrane by observing the absorption of heavy water (deuterium oxide—D_2O) from stomach pouches in the dog. One animal had a pouch of the fundus, or acid-secreting portion of the stomach and another, a pouch of the gastric antrum. Under the conditions of the experiment, half of the D_2O was absorbed in approximately 20 minutes. There was no significant difference between the body and the antral pouches in the rate of absorption. Whether the body pouch was in a secreting or resting state also made no difference in absorption. If it is assumed that the gastric membrane handles D_2O as it does H_2O, it can be concluded that there is a rapid exchange of H_2O between the gastric contents and body fluids.

ACIDS

That the absorption of HCN from the stomach is rapid was reported by Meltzer (76) who found that in unstarved rabbits weighing about 2.2 kg., the introduction

of 60 mg. and 100 mg. of HCN (as the salt) into stomachs ligated at cardia and pylorus resulted in death in 12 and 15 minutes, respectively. Meltzer felt that the absorption of HCN may have been due to diffusion of the highly volatile HCN (in acid gastric juice) and/or the production of a hemorrhagic surface on the mucous membrane, which would facilitate absorption.

Regarding other inorganic acids, Teorell (105a) in 1933 showed that in decerebrate or anesthetized cats the stomachs of which were tied off at both pylorus and cardia, with a cannula being inserted into the pyloric end, a very active disappearance of HCl occurred and that this absorption of the acid was a consequence of rapid diffusion of the hydrogen ion into the gastric mucosa. Although Klimov and Kudriavtsev (55) found that natural gastric juice and 0.2 per cent HCl can be absorbed to the extent of 35 per cent to 45 per cent in calves and Feitelberg (31), likewise, found natural gastric juice to be absorbed by the stomach of the dog, Hollander (48a) and Wilhelmj (113a) in their studies on gastric secretion came to the conclusion that absorption of HCl from the stomach could not have occurred as stated by earlier workers. Support to the latter view was rendered by Shay *et al.* (94a) who were unable to find evidence of absorption of 0.5 per cent to 1.0 per cent HCl in 30 minutes from human stomachs. Nevertheless, Teorell (105b) again reported that the gastric mucosa of the cat was permeable to both weak and strong acids.

Of the experimental data presented by the proponents and the opponents of the theory that the gastric mucosa is permeable to inorganic acids, the most cogent arguments are to be found among the proponents. Added support to the validity of the data and conclusions regarding positive gastric permeability to acids is afforded by the experimental results with both volatile and non-volatile organic acids reported in the following paragraphs.

Inouye and Kashiwado (50) concluded that the gastric mucosa of the dog absorbs salol (phenyl salicylate) and salicylic acid. They stated that it is probable that not salol but its hydrolytic product, the acid, is actually absorbed from the stomach. Prior to the work of these authors, Stein (102) had also reported the permeability of the stomach to salol. Confirmation of the gastric penetration of salicylic acid came from Carnot, Papaconstantinou and Simonnet (15) who reached the conclusion that absorption of sodium salicylate was positive when the stomach contents were acid and negative when the gastric contents were neutral or alkaline. The absorption in the acid stomach was so rapid that salicylate was detected in serum from the third minute after the salt was introduced into the dog stomach ligated at both ends. The total amount absorbed was about 30 per cent of 0.50 gm. given as a 10 per cent aqueous solution. The serum concentration of salicylate was increased when the stomach was in the fasting state and when there were gastric lesions (gastritis) in the mucous lining.

Studying the absorption of volatile acids from the rumen of sheep, on the basis of the acid content of blood drawn from veins of the rumen, McAnally and Phillipson (73) introduced sodium acetate, propionate and butyrate into fistulas in the rumen. They found that introduction of sodium acetate into the fistula caused an immediate increase in the concentration of volatile acid in the blood from the rumen; whereas an equimolecular solution of the butyrate caused no significant increase. This observation suggested that the rate of absorption depends on the size of the molecule.

Continuing the study of absorption of volatile acids, Barcroft, McAnally and Phillipson (3) reported that when 25 grams of acetate in 2 liters of H_2O and equimolecular concentrations of propionate or butyrate were introduced into the rumen of sheep, the acetate and propionate appeared in the blood within five minutes; while the butyrate was hardly at all absorbed within this period. Positive results similar to those obtained in the sheep were obtained in the pig also.

In the rabbit and pony, however, Barcroft *et al.* (3) were unable to demonstrate absorption of acetate, propionate or butyrate; and in the dog, Klemperer and Scheurlen (54) were unsuccessful in their attempts to find evidence of absorption of oleic acid.

ALCOHOLS

Much of the work relating to absorption from the stomach has been conducted with ethyl alcohol. Regardless of the techniques and animals employed, there has been essential agreement concerning the significant disappearance of ethyl alcohol by virtue of its ability to penetrate into the stomach wall (7, 11, 12, 16, 22, 28, 31, 32, 40, 55, 81, 93, 104, 105, 112). Only the data of Rasmussen (87) and of Dybing and Rasmussen (25) obtained on rats indicate that absorption of alcohol is insignificant and these workers attribute the poor absorption to the fact that the stomach of the rat differs from that of other species in that two thirds of the rat stomach consists of a membrane which has a multi-layered, glandless, relatively impermeable, 'horny' epithelium. However, Haggard, Greenberg and Lolli (38) and Karel and Fleisher (53) have offered evidence of the consistent and significant absorption of ethyl alcohol in this species.

Investigations of the gastric absorption of alcohols other than ethyl have been limited to methyl alcohol, shown by Haggard *et al.* (38) to be rapidly absorbed, and to propylene glycol. Lehman and Newman (58) introduced 6 cc. of propylene glycol per kg. of body weight into the isolated stomach of the dog and found, at the end of 120 minutes, that the blood propylene glycol was only about 1.0 mg. per cent. However, according to van Winkle's data (109) when 10 cc. of 10 per cent propylene glycol per kilo of body weight was injected into the ligated stomachs of cats and rabbits, about 8 per cent was absorbed in 10 minutes, about 18 per cent in 30 minutes, 21 per cent in 60 minutes and 30 per cent in 120 minutes. Rats showed a somewhat more rapid absorption of the glycol than cats and rabbits, but the quantitative relations were similar.

ALKALOIDS

Confirmation of the original observations by Magendie (71), that strychnine can be absorbed from the ligated stomach of the dog, has come from several investigators and has been extended to include other species as well. Thus, Colin and Bouley (17) were able to demonstrate in dogs that death could occur within five minutes after the injection of 5 to 7 grams of an alcohol extract of nux vomica into the ligated stomach, although alcohol itself apparently did not significantly increase permeability (13, 76, 77). Similar results were obtained by Colin and Bouley (17) in the rabbit and pig; in the cow, spasms did not occur until $4\frac{1}{2}$ hours after the injection of 32 grams into the fourth stomach (abomasum), while in horses even 32

grams produced no symptoms in 35 hours. Negative results on the horse were also obtained by Perosino *et al.* (84) and by Berard (6) with 5 grams of strychnine sulfate.

In cats, according to Dixon (23), injection of strychnine into the ligated stomach was followed by symptoms of poisoning in 30 minutes.

In rabbits, although active absorption from the gastric cavity was demonstrated by Brooks (14), Meltzer (76) found that absorption of strychnine, with both cardiac and pyloric ends ligated and with blood vessels carefully excluded, did not occur even after 19 hours with a dose of 60 mg., whereas, when the drug was injected into the stomach wall, the tetanic effects were quickly evident. Even 60 mg. in 10 cc. of a 40 per cent alcohol solution given to a 0.98-kg. rabbit fasted for three days produced no effects in 15 hours. Meltzer (77), in a subsequent paper, reported that in dogs with pylorus and cardia ligated, $2\frac{2}{3}$ mg/kg. of strychnine had no effect in $2\frac{1}{4}$ hours and 3 mg. had no effect in 2 hours, but 20 mg/kg. caused tetanus in 42 minutes. The absorbent power of the fundus seemed to be even lower than that of the entire stomach. Likewise, Inouye and Kashiwado (50) were unable to demonstrate absorption even after 50 hours following the introduction of 5 cc. of atropine in 1 per cent and 2 per cent solutions in fasted dogs or cats with both cardia and pylorus ligated, when the end point used was dilatation of the pupil, although the unabsorbed drug was found active when placed in the eye of a test animal.

Using goats, Trautmann (108) prepared fistulae in the paunch, reticulum and abomasum, and inserted cannulae into the fistulae. Atropine and pilocarpine were found to be rapidly absorbed from all three compartments.

In a study of the influence of the hydrogen ion concentration on the absorption of alkaloids from the stomach, Travell (107) showed that in the cat and dog, with ligated cardia and pylorus, alkaloids, including atropine, were not absorbed to any extent from the stomach when the reaction of the gastric juice was strongly acid. But, when the gastric juice was rendered alkaline with $NaHCO_3$, alkaloids were rapidly absorbed from the ligated stomach. Similar results were obtained with cocaine, nicotine, strychnine and physostigmine. In the case of strychnine, the rate of absorption varied in general with the *p*H and the dose, decreasing with increase in acidity. Observations were conducted on the ligated organ for as long as 24 hours or more.

However, previously, when Ryan (89) introduced about one mg/kg. of strychnine into a miniature Pavlov pouch of such nature that the alkaloid was limited to the gastric mucosa, and the acidity of the stomach was 0.35 per cent in terms of HCl, a sufficient amount of a 0.5 per cent aqueous solution of the nitrate was absorbed in nine minutes to cause changes in the reflexes of the animal.

Although the data from the several investigations tend to confuse the picture regarding the inherent rôle of *p*H in gastric permeability, it is, nevertheless, clear that sufficient absorption of alkaloids may occur to produce toxic symptoms in the animal.

CARBOHYDRATES

Perhaps the most controversial issue in the field of gastric absorption is the question of the permeability of the stomach to carbohydrates. Aligned on the side of positive absorption of glucose are Brandl (12), Delhougne (22), Edkins (27),

Freund and Steinhardt (36), Holtz and Schreiber (49), Klemperer and Scheurlen (54), Segall (93), Smith (97), Strauss (103), Tchekounow (105), Teorell (105b), von Anrep (111) and von Mering (112); and, regarding inulin, Heupke and Blanckenburg (47). However, only the data of Edkins (27), Klemperer and Scheurlen (54), Smith (97) and Teorell (105b) appear to be clearly unequivocal.

London and his co-workers (60, 62, 64), on the contrary, are in complete disagreement, stating that carbohydrates, including glucose, sucrose, starch, amylodextrin and erythrodextrin definitely are not absorbed by the stomach of the dog. Confirming the latter data on the impermeability of the gastric mucosa of the dog to carbohydrates is the more recent report of Maddock and his colleagues (69). Fundamentally in agreement with the previously enumerated authors who believe that sugars cannot penetrate the mucosa of the normal stomach are Fenton and Pierce (32a) and MacLeod *et al.* (68) whose investigations were conducted with rats, and Martini *et al.* (72), who worked with dogs. All three groups present data indicating that while absorption may occur under certain conditions (see below), even when it does occur, it is not appreciable.

MacLeod, Magee and Purves (68) determined the extent of absorption of glucose in the stomach of rats, using the technique of ligating the pylorus, but leaving the esophageal opening to the stomach untouched. To prevent regurgitation, the esophagus was ligated before excising the stomach. The animals, anesthetized with urethane, were given 1 to 2 cc. of glucose solutions varying in strength from 14 to 51.5 per cent. The time of contact between glucose solution and gastric mucosa was varied between 0.1 hour and 2 hours. No appreciable absorption was found. The glucose recovered ranged from 95.5 per cent for 1 cc. of a 20 per cent solution left in the stomach for 1 hour to 99 per cent for 1 cc. of a 51.5 per cent solution left in the stomach for 0.1 hour. There was no apparent correlation between concentration of glucose solution, time of sojourn in the stomach and percentage absorbed.

Maddock, Trimble and Carey (69) measured absorption by determining the sugar content of blood from the gastric veins. Amytal was used as the anesthetic, since it has no hyperglycemic effect. As it is known that losses of gastric secretions may occur under anesthesia, these authors confirmed their experiments conducted with the animal under anesthesia by a preliminary surgical procedure which enabled them to close the pylorus after the animal had fully recovered from the operation. At the beginning of the absorption experiment, the pylorus was blocked and a solution of glucose was introduced into the stomach. After a known interval, the animal was killed by amytal, the stomach was removed, its contents were collected and the sugar present was determined. The esophagus was ligated at its upper end before recovery of glucose from the stomach was attempted. The blood sugar was measured by the microferricyanide method of Folin and Malmros. The determinations of blood sugar were made in samples—drawn 20, 40 and 60 minutes after the sugar solution was given to the animal. The authors stated that the sugar content of blood from the small veins of the stomachs of 4 dogs was not definitely in excess of the probable errors of sampling and of measurement when amytal anesthesia was used. In six experiments without anesthesia, where absorption was determined by the difference between the amount given and the amount remaining, the recovery

of sugar one to two hours after its introduction averaged 99.3 ± 1.2 per cent of the glucose administered. In two control trials, 99.1 ± 0.2 per cent was recovered immediately after its introduction had been completed. The quantities given ranged from 5 to 47 grams in concentrations ranging from 2.8 to 38 per cent. These results were interpreted by Maddock and his colleagures as demonstrating that the absorption of glucose in significant quantities from the stomach of the dog does not take place.

In 1936 Feitelberg (31) conducted a series of studies on 2 dogs with isolated Pavlov stomachs. The stomachs contained a metal fistula tube and the pouches were formed from the fundic portions of the gastric cavity. The tests were made on an empty stomach following 16 to 18 hours of fasting. The carbohydrates tested were introduced through the fistula tube and were left in the stomach for 1½ hours. The strength of the solutions varied between 5 and 30 per cent and the amount introduced ranged between 20 to 30 cc. The solutions were warmed to 38° C. prior to their use. Recoveries were estimated by means of a Zeiss-Volny refractometer. Blood sugar both before and after introduction of the carbohydrates into the gastric cavity was estimated by the Hagedorn-Jensen method. The results obtained by Feitelberg were as follows:

The absorption of glucose begins only with the introduction of solutions of 10 per cent or greater, the higher concentrations producing somewhat greater absorption, although the amount of absorption after one hour is not significant. As would be expected, the longer the sojourn in the stomach, the greater the absorption. In one experiment, 25.95 per cent of 27 cc. of an 11.8 per cent glucose solution was absorbed in 120 minutes. Controls to determine the extent of decomposition of sugar in the stomach were negative after 12 hours contact of gastric mucus secreted for 2 hours from the isolated stomach and glucose in Hiss' Medium. The resorption of galactose was found to be considerably higher than that of glucose, 23.98 per cent of 22 cc. of a 21.5 per cent solution being absorbed in one hour and 40.64 per cent in two hours. Blood sugar increase could not, however, be detected. In solutions weaker than 22 per cent, levulose was not absorbed as well as glucose. With 22 to 25 per cent solutions, the absorption almost equalled that for glucose. Again, hyperglycemia—as in the case of glucose—was not demonstrable. The resorption of lactose was poorer than that of galactose and was almost equal to that of glucose. No increase in blood sugar could be detected for the solutions used, which ranged from 6 to 20 per cent in strength. There was no absorption of low concentration sucrose solutions in one hour, but increasing the concentrations to 38 per cent markedly increased absorption. However, the absorption of sucrose was poorer than that of other sugars; as in the previous instances, blood sugar increase could not be detected.

Resorting to the technique of closing the pylorus by duodenal stimulation, Shay *et al.* (94) studied the absorption of glucose in the stomach, as affected by duodenal stimulation by HCl, olive oil, $NaHCO_3$, saline and glucose. Five minutes after the duodenal instillation of the stimulant, 225 cc. of about 40 per cent glucose mixed with 2 ounces of $BaSO_4$ were ingested. The instillation was continued for 30 minutes. Fluoroscopic observations and roentgenographic records were made at

frequent intervals in order to make certain that none of the gastric contents had left the stomach. In addition, duodenal contents were checked for sugar every 10 minutes. On the basis of their data, these authors concluded that glucose, at least when in high concentration, may be absorbed by the human stomach. In a subsequent paper on the absorption of glucose from the human stomach, Shay *et al.* (95) again concluded that considerable amounts of glucose may be absorbed by the stomach alone from solutions of high concentration. From concentrations below 15 per cent, no appreciable absorption could be demonstrated.

Determining the absorption of glucose one hour after its introduction into the ligated stomachs of dogs, Morrison *et al.* (79) found that glucose is absorbed from the stomach when present in high concentrations and that the rate of absorption seems to bear a relationship to the concentration of the solution in the stomach.

In 1940, Rankin (86) working with sheep reported that when 2 molar dextrose was placed in the isolated rumen in a ratio of 4 gm/kg. body weight, the absorption of dextrose as determined by increase in blood sugar levels was marked.

By using a modification of the balloon technique to close the pylorus, Warren *et al.* (113) were able to study the absorption of glucose in the human stomach. They stated that when concentrated glucose solutions were ingested, a certain amount left through the stomach wall during the first short period of contact. The indirect evidence was supported by experiments in which the pylorus was mechanically closed. The strength of the glucose solutions varied between 2.46 and 60.3 per cent and the interval between injection of the glucose through the stomach tube and withdrawal of samples by washing varied from 4 to 37 minutes. In no instance was more than approximately 13 per cent of the sugar absorbed.

In general, the following conclusions may be drawn from the data of the authors mentioned: *a*) Little, if any absorption of glucose or sucrose from solutions of low concentrations (<15%) occurs during the first hour or two; *b*) where the concentration of glucose, sucrose and some other carbohydrates is greater than about 20 per cent, absorption for some, but not all, can be decisively demonstrated, particularly when contact between carbohydrate solution and gastric mucosa is greater than one to two hours (when concentrated solutions are used, however, much of the permeability may be the consequence of mucosal congestion produced by the hypertonic solution (cf. Brandl)); *c*) often, although the data are acceptable, an insufficient number of experiments has been reported to justify general conclusions; and *d*) species variation may play an important rôle in the divergence of results.

FATS

In 24-hour fasted dogs in which the pylorus and cardia were ligated, Klemperer and Scheurlen (54) found that olein was not absorbed in three or in six hours. A similar conclusion was reached by Volhard (110), who reported that unsplit egg and milk fat does not permeate the stomach even after contact of two and one-half hours, and by Delhougne (22), who found no absorption of triolein in three hours. While the work of both Volhard and Delhougne is subject to the criticism that some of the experimentally employed fat may have escaped into the intestine, the negative results obtained even with the possibly faulty technique strengthen the conclusions of

Klemperer and Scheurlen. On the other hand, Schilling (92) reported that in calves fed fats the gastric mucosa was thickly imbedded with fat droplets, thereby indicating absorption. The droplets were found not only in the epithelium, but also in the tunica propria and in the parenchyma. A similar observation regarding the plaice (a member of the flounder family) was made by Dawes (21), who likewise resorted to histological examination as evidence of absorption.

Following earlier experiments by Baumann (5), Mendel and Baumann (78) asserted that, although from the histological findings it appeared that an increase in the amount of fat in the gastric mucosa and submucosa resulted when fat was ingested and that the appearance strongly resembled the microscopic picture obtained during intestinal fat absorption, their experiments on cats and dogs, in which the stomach was ligated at the pyloric end and in which they followed fat content of the blood and traced oil soluble dyes, indicated that no active absorption of fat occurred from the ligated stomach. The materials used were cream, peanut oil emulsion (peanut oil—45%, lecithin—5%, water—50%), olive oil and Sudan III stained fat. Experiments continued for 6 to 12 hours during which the thoracic and common bile ducts were cannulated, but no fat was ever found in the lymph channels or in the portal circulation.

Although the weight of the currently available evidence (22, 54, 110) is such as strongly to suggest the validity of the statements made by Mendel and Baumann (78), this evidence does not refute the histological. Rather, it merely implies that not enough fat is absorbed to yield positive proof of its absorption by blood examination. Were it possible to sensitize animals to the various fats as it is to proteins, it might be found by sensitization phenomena that some fats are able to penetrate the normal mucosa.

PROTEINS AND PROTEIN CLEAVAGE PRODUCTS

From experiments with the ligated stomachs of cats and dogs into which, at different times, peptone, taurine, albumoses, acid-albumin (*syntonin*), fibrin and glycine were introduced and allowed to remain in contact with the stomach tissues for periods which varied from 30 minutes to 4 hours, depending on the investigator, it was concluded by Brandl (12), Delhougne (22), Lang (57), Salaskin (90), Tappeiner (104), Teorell (105b), Tobler (106), von Anrep (111), von Mering (112) and Zunz (114) that the gastric mucosa is permeable to proteins and their cleavage products—although Delhougne also reported that no absorption of glycine occurred prior to $2\frac{1}{2}$ hours and that even at 4 hours, peptone remained unabsorbed. With the exception of the work by Tappeiner (104), Teorell (105b) and Zunz (114), however, the likelihood of leakage into the highly absorptive duodenum was present in the studies reported.

In regard to other species, Smith (97) published data which was indicative of marked absorption from the ligated frog stomach, while Scheunert (91), whose findings are questionable, stated that the absorption of protein in horses was to a degree dependent on the digestibility of each individual substance and was comparable to that in the dog. Klimov and Kudriavtsev (55), using stomachs isolated by the method of Heidenhain, found definite gastric permeability of glycine in bull

calves; Davey (20), on the basis of osmotic data obtained by the freezing point determination procedure of Beckmann, suggested that absorption occurs from all four stomach compartments of the sheep (reticulum, rumen, omasum and abomasum), although only the abomasum has a lining epithelium of secretory cells and is comparable with the stomach of the non-ruminant.

Using stomach and pyloric fistula animals, London *et al.* (60), London and Sulima (61) and London and Polowzowa (62, 63, 65, 66) emphatically disclaimed the likelihood that there could be any absorption of proteins or protein cleavage products by the non-pathologic canine stomach; although in an earlier investigation, Abderhalden, Prym and London (2) made the statement that absorption, if it does occur, is slight; and in part of London's subsequent data there is evidence, which the authors choose to doubt, that absorption, though small in extent may, nevertheless, occur. The various substances investigated included egg albumen, glycine, l (+) alanine, dl-leucine, casein, serum, gelatin, glutamine and edestin. Comparable results with glycine were achieved by Martini *et al.* (72), who employed the Pavlov pouch technique in dogs.

Following heated controversies during which many laboratories were overtly involved in the question of protein permeability, Folin and Lyman (34, 35) reported on their work with cats in which ligatures were placed at both the cardiac and pyloric ends of the stomach. After the substance to be tested was injected into the gastric cavity, the abdomen was closed. At suitable intervals, 5-cc. samples of blood were taken from the carotid and femoral arteries and analyses for digestion products were made. Also, at the end of the experiment, splenic and enteric tributaries of the portal vein were ligated and a sample was taken from the portal vein which was by this means robbed of all but its gastric branches. They found that the non-protein nitrogen of the blood steadily increased (up to about 180 minutes after injection) for the following, while the urea nitrogen remained constant: glycine (61 gm. in 40 cc. of warm water were injected into the ligated stomach), alanine (6.4 gm. in 50 cc. of warm water were injected), Witte's peptone (8.0 gm. in 40 cc. of warm water were injected). With creatinine, the N.P.N. did not increase up to 5 hours and 10 minutes after injection of 2.8 grams in 50 cc. of warm water. The creatinine was quantitatively recovered. The experiment was repeated with the same result, showing no absorption of creatinine. When 1.8 grams of urea in 25 cc. of warm water were tested, it was found that urea was absorbed. The earliest time of increase in N.P.N. for the various substances was alanine—30 minutes, Witte's peptone—30 minutes, glycine—38 minutes and urea—90 minutes.

Recently, Harten *et al.* (42) used a qualitative test of absorption dependent on the prior sensitization of a cutaneous site to the antigen to be studied. The urticarial reaction at the sensitized cutaneous site within a few minutes marked the entrance of unaltered protein into the circulation. In this manner, the absorption of unaltered cottonseed protein was experimentally demonstrated to occur from the esophagus and from the fasting, ligated stomach in the rhesus monkey, the absorption time from the stomach having been 11 minutes in one monkey and 2 minutes in another.

A critical interpretation of the physiological and the chemical analytical methods

employed in the experimental evaluation of the absorption of proteins and their cleavage products from the stomach inevitably leads to the conclusion that not only may absorption occur, but that it can occur quickly following ingestion.

GASES

With the exception of the conclusions in the paper of Bassal and Uteau (4) cited below, which may be discounted, it is agreed that gastric absorption of gases may occur. Von Mering (112) noted that when soda water was introduced through a fistula into the stomachs of dogs, H_2CO_3, presumably as CO_2, was absorbed in large amounts. Much later, Edkins and Murray (28) stated that in decerebrate cats, with esophagus ligated and pylorus cannulated, the rate of absorption of CO_2 was increased in the presence of alcohol and vice versa. In 1926, McIver, Redfield and Benedict (75) reported that a study of the behavior of CO_2 in the stomach, as a typical case of gaseous exchange, resulted in strong evidence that the movement of the gas into and out of the stomach was not due to secretory factors, but was governed by the physical laws of diffusion (cf. von Mering, 112). The gas to be studied was introduced into the stomach, ligated at the cardia and pylorus, of cats fasted 24 to 48 hours but allowed free access to water. The stomach was washed out with warm water before the experiment and the body temperature was measured and maintained by heating pads. They found that of 50 cc. of O_2 introduced, 6.7 cc. were absorbed in one hour; while 19.4 cc. were absorbed in three hours. The absorption of CO_2 was greater, 39.0 cc. of the 50 cc. being absorbed in 15 minutes. On the other hand, N_2 was absorbed only to the extent of about one cc. in one hour. One hour after the introduction of 60 cc. of air, the analysis of the gas withdrawn was CO_2—5.2 per cent, O_2—15.3 per cent and N_2—79.5 per cent.

In 1912 Bassal and Uteau (4) ligated the cardia and pylorus of guinea pigs and rabbits. Results with $CHCl_3$, $(C_2H_5)_2O$ and CO were negative for periods of 20 to 25 minutes, but the analytical data are questionable.

Singh (96) estimated from his experiments in which the pylorus was not ligated that the possible maximum rates of absorption of O_2 from the stomach were 0.2 to 0.4 cc/min. as measured from change in oxygen consumption through the lungs.

According to Fine, Sears and Banks (33), when the ligated stomach of a cat breathing O_2 was distended with N_2, the total gas volume was as a rule about as great after periods varying from 6 to 24 hours as at the beginning of the experiment. When H_2 was used instead of N_2, the total gas volume was reduced about 10 per cent after 12 to 24 hours. Since other gases diffused into the stomach until an equilibrium was established, it appeared that on the basis of gas analyses, both N_2 and H_2 were absorbed from the stomach.

DYES

Kobayashi (56) ligated the cardia and pylorus of dogs by the standard technique in which the blood vessels leading to and away from the stomach are left intact. To determine positively that any dyestuff not recovered from the stomach contents had been absorbed rather than adsorbed, he collected control samples of bile, urine and serum. Then, 50 cc. of 0.5 per cent dye solution was introduced by a thin needle

into the stomach and, to compensate for loss of body fluids withdrawn as samples, an equivalent volume of saline was injected subcutaneously. Every hour, bile, urine and serum were collected for comparison with the controls. Each animal was observed for more than eight hours. At the end of an experiment, the animal was sacrificed and the stomach contents were analyzed. Kobayashi concluded that, in general, a dyestuff which has great diffusibility can be absorbed by the stomach, whereas a dyestuff which has little diffusibility cannot be absorbed by it. This fact is not influenced by many conditions, for example, acid or base reaction, chemical structure, solubility in lipoids or vital staining. Diffusibility was determined by placing 0.5 per cent dye in agar-agar gel and measuring the diffusion of the dye into the gel for an eight-day period. Those dyes, according to Kobayashi, having a diffusibility greater than 5.5 cm. can be absorbed by the stomach. Those with less than 5.0 cm. cannot be absorbed. In all, 31 dyes were investigated, including mono- and diazo-triphenylmethane, xantheine, azine, oxazine and thiazine derivatives. It is regrettable that data for periods much less than the selected observation period of eight hours are not given.

Several years later, Henning (45) conducted a series of experiments in which he allowed one per cent aqueous dye solutions to remain in contact with the gastric mucosa of the frog for, usually, 5 minutes. At the end of this period, the dye was washed off with Ringer's solution. If a dyeing of the cells was not microscopically evident, the dye was permitted to remain in contact with the stomach for 20 minutes. *Uranin*, a fluorescent dye, was strongly absorbed, especially in the uppermost portion of the glands. The rapidity of absorption was such that one minute after wetting the mucosa, the dye could be detected, by its fluorescence, in the plasma. *Eosin*, *erythrosin*, *magdalarot* and *trypan* blue, like *uranin*, could be ultimately detected in the blood. On the other hand, *Chicago blue* and *diamine black* did not dye the cells of the stomach even after contact of 30 minutes, indicating no absorption. Several basic dyes investigated, like *malachite green* and *alizarin blue*, were readily absorbed, selecting the edges and lacunae, but could not be found in glandular or interstitial cells. There was some absorption of *methylene blue* and *congo red*, but other dyes like *neutral red* and *acid violet* were not absorbed. This author also stated that the chemical relationship of the individual dyes cannot be the basis of their similar biological behavior, inasmuch as compounds of entirely different chemical constitution dyed similar cell-complexes.

IRON

Although Hári (41), using reduced iron, at first found that even after $3\frac{1}{4}$ hours there was no absorption of iron as determined histologically, he subsequently obtained positive results at the level of the fundus and the pars pylorica in one experiment in which about one gram was administered; in four experiments in which 0.2 gram was given, absorption from the fundus and from the pars pylorica was positive three times.

Later, in 1900, Abderhalden (1) investigated the absorption of inorganic iron in rats, rabbits, guinea pigs, cats and dogs. Using microchemical-histological methods, he concluded that no iron was absorbed by the stomach during experiments

lasting 24 to 120 hours. Hofmann (48) was also unable to show by histological means that iron is absorbed from the stomachs of human beings or of guinea pigs.

However, in 1928, Starkenstein (99), after working with frogs, rabbits and rats, stated that all parts of the gastro-intestinal tract are capable of the absorption of iron. Finally, in 1943, Hahn *et al.* (38a), employing radioactive iron, were able to show that very active absorption of Fe could occur from a gastric pouch of a completely isolated stomach of a dog. While only one animal was used in the experiment of Hahn and his colleagues, the evidence is such that the successful repetition of their findings is quite likely. Meanwhile, in simultaneous studies not reported until a year or so later, Vahlquist *et al.* (108a) established that in individuals with artificial stenosis of the pylorus iron is absorbed from the stomach in rabbits and in man. Significant increases in serum Fe^{++} following the administration of the lactate occurred in all subjects in 45 minutes or less.

MISCELLANEOUS

While much of the data on the absorption of salts may be criticized because of inadequate control observations, there is, as with alcohol, no fundamental disagreement regarding the permeability of the gastric mucosa to salts. Moreover, enough sound positive evidence (cf. 17, 19, 29, 39, 43, 55, 74, 86, 105b) is available to obviate doubts concerning the absorption of salts in general and of inorganic salts in particular.

In 1880, Tappeiner (104) asserted that although Na_2SO_4 could be absorbed, of 1.57 grams of the salt introduced into the ligated stomachs of cats and of dogs, only about 16 per cent had disappeared from the stomach after $3\frac{1}{2}$ hours, whereas chloral hydrate was absorbed only from alcoholic solution and not at all from its aqueous solution. Subsequently, absorption of salts, among them NH_4Cl, NaCl, $CaCl_2$, $NaHCO_3$, Na_2CO_3, CH_3COONa, KI (or NaI) and KBr were reported by Bönninger (10), Brandl (12), Jaworski (51), Klimov and Kudriavtsev (55), Roth and Strauss (88), Teorell (105b) and von Mering (112). More recently, McDonald (74a) has shown that absorption of ammonia occurs from the rumen of sheep.

Brandl (12) noticed that in all instances where absorption had occurred, the gastric mucosa was measurably erythematous. After investigating the effect of several stomachics on the gastric permeability of aqueous solutions of different substances, he concluded that the increased intragastric penetration was probably the consequence of irritation of the mucosa.

On the basis of positive urine tests following the introduction of 0.5 gram quantities into the stomach, Delhougne (22) stated that the absorption of KI and of sodium salicylate had occurred within two hours, while that of KBr, pyramidon and quinine had occurred within one hour.

Following the study of Starkenstein and Hahnel (100) on factors affecting the absorbability of magnesium salts, Hay (43) studied the absorption of saline cathartics in the cat and concluded that Na_2SO_4 was absorbed to a slight but significant extent. Jaworski (51), using his previously described technique, listed the decreasing order of absorbability as *a*) $H_2Mg\ 2\ (CO_3) > NaHCO_3 > Na_2SO_4 > Mg\ SO_4 > NaHPO_4$

> KCl > $FeCl_2$ > NaCl and *b*) Na_2SO_4 > Na_2CO_3 > $MgCl_2$ > KCl > $FeCl_2$ > NaCl.

Otto (82) reported only negligible absorption of $MgSO_4$; according to Myant (80) loss of SO_4 in the quiescent stomach of the cat cannot be observed when the concentration of the solution is 4 per cent or less. Henning (44) stated that the normal mucous membranes of the human stomach are impermeable to iodide ion in aqueous solution but are permeable to iodide in alcoholic solution. Hanzlik (39), however, found that marked absorption of NaI in water solution occurred from the ligated stomachs of dogs and of cats after one-half hour.

In the experiments of Eisenman *et al.* (29), isotonic solutions of radioactive isotopes of Cl^-, Na^+ and K^+ were introduced into the stomach of rabbits with ligation at the pylorus. Sometimes non-radioactive solutions containing Br^- and I^- were introduced as well. In a parallel series of observations, solutions were placed in an isolated gastric pouch of the stomach of a dog. By comparison of the specific radioactivity of serum or tissue at the end of an hour with that of gastric juice, an estimate of the degree of absorption was obtained. Na^+, K^+, Cl^-, Br^- and I^- placed within the stomach were all transferred to some extent across the stomach wall into the general circulation. The transfer after one hour, however, was too slight to permit an approach to equilibrium. The transfer of Na^+ and K^+ was much slower than that of Cl^- which, in turn, was slower than that of Br^- and I^-.

In 1943, Cope, Cohn and Brenizer (19) reported on the absorption of radioactive Na^+ from pouches of the body and antrum of the stomach of the dog. They found that the absorption of radioactive Na^+ as NaCl within 15 minutes from the body or acid-secreting area was small but significant. Two to three times as much was absorbed when the mucosa was in the resting rather than in the secreting state. The gastric antrum absorbed 100 times as much per unit of surface area as the acid secreting body. Whether the stomach was fasting or secreting made less difference to the absorption from the antrum than to absorption from the body. Variations in the osmotic pressure of the Na^+ solution and in electrolytic concentration in the blood serum, within the limits observed, had no significant effect on the rate of absorption of the Na^+.

According to the data of Rankin (86), when 4 grams of KI were dissolved in 200 cc. of warm water and placed in the rumen of sheep, the saliva gave a positive test for I^- within 5 minutes. Methylene blue which had been mixed with the iodide indicated complete localization of the latter. Using the same technique with NaCN, Rankin gave 0.4 gram dissolved in 200 cc. of saturated methylene blue. In 20 minutes, the animal showed labored respiration with its head extended and tongue blue and, in 43 minutes, the sheep was dead. It may be recalled that in these experiments, the rumen was exposed by fistula and isolated from the other compartments.

Colin and Bouley (17) reported that the stomachs of the dog, the cat and the pig absorb $K_4Fe(CN)_6$ while those of the deer and the horse do not.

By means of radiographs of the bladder before and after dosing, McAnally and Phillipson (74) determined that the radio-opaque salt sodium ortho-iodo hippurate, when administered in the quantity of 2 gm/kg. body weight into the isolated rumen, was absorbed, although absorption was slow. A distinct bladder shadow was ob-

tained one hour after administration of the hippurate, but was not dense until the fourth to fifth hour.

Sollman, Hanzlik and Pilcher (98) introduced one gm/kg. body weight of phenol into the doubly ligated stomachs of cats and dogs. Even after several hours, a large proportion could be removed by gastric lavage. Dunn and Perley (24), working with rabbits in which the pylorus was open, concluded that the effect of alcohol on the gastric absorption of phenol was slight. The appearance time of convulsions and the time of death were essentially the same with or without alcohol.

Levyn and Beck (59) introduced 3 gm. of tetraiodophenolphthalein into the Pavlov pouch of a dog weighing 48 pounds. Seven, 17, 48 and 53 hours later roentgenograms were made of the gall bladder, but no shadows were obtained in any of the trials. Introduction of 3 grams of tetraiodophenolphthalein in 30 cc. of water and of 8 grams of dye in 60 cc. also gave negative results; the latter two doses placed into the duodenum, however, yielded positive results.

Shortly after the appearance of the report by Penner and Hollander (83) on the non-absorption of phenol red, Shay *et al.* (95) confirmed the fact that phenol red is neither adsorbed nor absorbed by the human stomach.

SUMMARY

It is unfortunate that many investigators who employed the technique of ligating both cardia and pylorus in their studies of gastric absorption overlooked the possibility that increases in hydrostatic pressure resulting from complete ligation and introduction of relatively large amounts of fluid would tend to produce greater 'absorption' than might occur were the stomach contents allowed to remain at atmospheric pressure (cf. Karel and Fleisher, 53). Nevertheless, data so obtained are not invalid even though the essential comparative data are lacking, for similar increased hydrostatic pressure may occur from a variety of not only pathological circumstances but, also, normal conditions such as the ingestion of large quantities of food and carbonated beverages.

In all but a relatively few studies, absorption has been studied over periods of time which are abnormally long and during which, particularly in ligated stomachs, the possibility of the occurrence of some degenerative changes, perhaps, as a result of neurological disturbances leading to increased permeability, has not been excluded. However, where, under the circumstances of unduly prolonged experiments, no absorption of a particular substance was shown to occur, one may be reasonably certain that under normal, physiological conditions absorption of that same substance is very unlikely.

It is regrettable that almost no work has been done on the complete quantitative recovery of test materials placed intragastrically. Experiments have largely been confined to determining the amount of substance disappearing from the stomach and further proof of its absorption by detection of the original compound in blood or other body fluids. That work on complete recovery which has been done has been limited almost wholly to ethyl alcohol studies.

While the need for quantitative definitive studies on the extent of gastric absorption occurring during short periods of contact, for example less than 30 minutes, is

obvious, sufficient positive evidence has been presented to indicate that the importance of absorption from the stomach must not be lightly discounted. While the stomach is definitely not an absorptive organ in the same sense as the intestine and cannot be considered of especial importance in supplying the nutritional needs of the normal organism, its absorptive ability, particularly as regards substances physiologically active in minute quantities, has been grossly underestimated. Investigations into the relationship of chemical and physical structure to gastric absorption should yield rich rewards in the increased knowledge concerning allergenic phenomena, toxicology and rapidly effective, oral therapeutics.

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THE PHYSIOLOGY OF ADIPOSE TISSUE

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THE PHYSIOLOGY OF ADIPOSE TISSUE has received comparatively little attention by physiologists. Howell (1), in his textbook of physiology, comes to the conclusion that "it is to be borne in mind that we know little or nothing concerning the physiology of the tissue most directly concerned in the deposition of fat, namely the fat cells themselves." A recent review by Wells (2) on the anatomical and pathological aspects of adipose tissue is rightly named "The Neglected Subject." The neglect is mainly due to the fact that adipose tissue has generally been regarded as an inert site of fat storage, without any specific metabolic activity. This point of view is, however, no longer tenable in face of data assembled during the last 20 years. The present review will endeavor to compile data concerning the specific physiological activity of adipose tissue and to show that the activity of this tissue constitutes an important link in the physiology of animal metabolism. Special consideration will be given to the more recent literature, in which references to older papers can be found.

CELL ORIGIN OF ADIPOSE TISSUE

The view that adipose tissue is merely ordinary connective tissue, in which fat has been deposited, was first expressed by Flemming (3) and is still generally taught, in spite of the fact that most recent histologists do not agree with this. Data obtained during the last 20 years show that adipose tissue develops from special primitive fat cells and that the cells of this tissue have a specific structure, entirely distinct from the fibroblasts of connective tissue.

The specific structure of adult adipose tissue has been clearly demonstrated by Maximow (4) in the case of the fat cells of the omentum. This specific, gland-like structure is not always evident in the fat-laden tissue and is restored only after depletion of the fat.

Direct proof of the occurrence of primitive 'fat organs' has been presented in a short note by Hausberger (5). Embryonic tissue, from a site that develops into adipose tissue, yields a typical fat tissue when transplanted into normal adult rats. Upon similar transplantation of embryonic connective tissue, no adipose tissue is formed. The embryonic fat tissue is fat free and is morphologically undistinguishable from embryonic connective tissue. The tissue is recognizable only by its specific location and development. Wassermann (6) demonstrated the affinity of fat tissue to reticular perivascular mesenchyme cells. The conception that adipose tissue is part of the reticulo-endothelial system is in harmony with the glandlike histological structure of the unfatted fat organ, as well as with the functions adipose tissue can fulfill along with its usual fat-storing function. Portis (7) showed that the cells of the omentum are able to form antibodies and that this ability is correlated with the

occurrence, in this adipose organ, of aggregates of reticulo-endothelial cells. Histologically, these cells closely resemble primitive adipose cells, before fat storage sets in. According to Wassermann (6), the blood-forming function may be reestablished in depleted adipose tissue under appropriate conditions. This is in analogy with the behavior of bone marrow, which sometimes adopts the function of fat storage in addition to its blood-forming function. Vital dyes are stored by adipose tissue just as in the reticulo-endothelium. This was shown by Dogliotti (8) in the depleted fat cells and by Bremer (9) even in the thin protoplasmatic ring about fat distended cells. McCullough (10) showed that Trypan blue is included in fat cells, especially after their depletion of fat. This is regarded as evidence for the macrophagial origin of adipose tissue. A critical review of the histological and embryological aspects of adipose tissue has been given by Wells (2).

It may be concluded that adipose tissue can no longer be regarded as connective tissue, fulfilling the function of fat storage. It seems to be part of the histiocyte system and is able to fulfill other tissue functions in addition to its specialization in fat storage.

INNERVATION OF ADIPOSE TISSUE

It had long been taken for granted that adipose tissue does not possess a nervous supply. Hofmeister (11), in a lecture in 1912, stated that fat mobilization is not governed by innervation, since no nerves are found in adipose tissue. The same attitude was adopted in a later research on brown fat tissue by Rasmussen (12). Goering (13), on the other hand, arrived at the opposite conclusion on the basis of clinical experience, which showed that localized fat deposition and depletion is governed mainly by nervous factors. Wertheimer (14) produced experimental evidence for the dependence of fat mobilization on the innervation of adipose tissue. The innervation of the blood vessels supplying the adipose tissue was demonstrated by Nordmann (15). This author believed that adipose tissue is influenced by the nervous system only through the vascular system. However, Hausberger (16) was able to show that the functional activity of the fat organs is regulated by an abundant nerve supply to both the vessels and the parenchyma. The best histological preparations demonstrating the nervous supply of fat cells are those of Boecke (17). In these preparations, fat cells of the interstitial tissue of the parotis are shown to be supplied by nerve fibers of the sympathetic nervous plexus of the surrounding tissue. It could not be proved with complete certainty, in a microscopic preparation, whether the nerve fibers penetrate the thin plasma membrane, but the curving of the fiber ends around the cell nucleus make this conclusion highly probable. Earlier histological papers, especially those of Stoehr and Reiser, are discussed by Boecke.

It may be concluded that adipose tissue is controlled in its activity by the nervous system.

BLOOD SUPPLY OF ADIPOSE TISSUE

The blood vessels of adipose tissue were the subject of several studies in the older literature (3, 6). Nordmann (15) tried to base a theory on the capacity of fat deposition and mobilization of adipose tissue on the sparsity of its blood supply. This

paper was later criticized by Hausberger (16), who concluded that Nordmann observed only the greater vessels and not the capillaries. The extent of the blood supply of adipose tissue was examined quantitatively in a recent paper by Gersh and Still (18). It was stressed that in the well developed fat sizes, generally examined by earlier authors, the capillary net appears to become coarser and inconspicuous, especially if the sections of the tissue are fixed in conventional ways. However, by a new method of fixing the fat and its contained blood by freezing and drying the tissue in unaltered condition or by arterial injection of the animal with India ink, the number and dimension of the capillaries present in the tissue could be estimated. It was found that the capillaries of the fat depots of the rat, unlike those in muscle, show no particular orientation, but form loose meshes which run in all directions in the tissue that encloses the fat cells. No marked quantity of fat cells escapes close contact with at least one capillary. The capillary density increases as the fat cells become smaller and there is considerable variation in the number of capillaries in different regions. The ratio of the surface of the capillary bed to the volume of the tissue supplied is about 52 in fat-rich tissue and about 220 in fat-poor tissue. The first value is about one-third as great as in muscle, the second is of the same order as in the most poorly supplied muscle. For metabolic purposes, however, the data are better calculated on the basis of surface area of capillaries to volume of protoplasma of fat cells. On this basis, a ratio of about 1000 is found in fat rich tissue for surface of open capillaries and of about 2160 for total capillary surface. This means that for metabolic purposes, the capillary bed of fat tissue is comparatively richer than that of muscle.

DEPOSITION OF FAT IN ADIPOSE TISSUE

The nature of the fat deposited in adipose tissue is determined by the balance between the fat in the diet, the fat synthesized in the body and that broken down in metabolism. Animals deposit a fat specific to their species, owing to their relatively specific feeding habits and because most of the body fat is that synthesized within the body, the diet of most animals consisting of predominantly carbohydrate foods. Only upon ingestion of large amounts of fat of specific composition is the fat store altered so that it approaches in composition that of the fat fed. An additional factor contributing to the constancy of fat composition is the ability of the body to carry out at least minor alterations in the fatty acids. It has been shown by Schoenheimer and Rittenberg (19) that upon ingestion deuteriostearic acid, deuteriopalmitic and deuterioleic acid are found in the fat deposited, proving the ability of the body to carry out desaturation, as well as shortening of the hydrocarbon chain. The site of these transformations in the body is not determined by these experiments. Previously it was generally accepted that adipose tissue does not play an active part in these processes. This can no longer be maintained, since the ability of adipose tissue to carry out desaturation of fatty acids has been repeatedly demonstrated (20–23). Moreover, the assumption that at least part of the desaturation is carried out in adipose tissue itself serves as a convenient explanation for the well known fact that the fat deposited in various parts of the body is not identical in its iodine value, the deeper layers being more saturated that the outer parts (24). The correlation between the temperature at the site of storage and the degree of saturation may be due to a de-

pendence upon the temperature of the equilibrium between saturated and unsaturated fatty acids in the fat body. An alternative explanation, proposed by Hilditch (25), that all the fat is synthesized and transformed in other tissues, especially in the liver, and that the adipose tissue at various sites selects a specific mixture of glycerides from the blood, also requires active work on the part of the adipose tissue. However, no experimental basis is as yet available to suppose such an elective ability.

The source of fat deposited can be divided into *a*) preformed fat from the diet and *b*) fat synthesized in the body from carbohydrates and proteins.

a) *Deposition of Fat from Preformed Fat.* Good evidence is on hand for the assumption that food fat is first deposited in the adipose tissue, after being absorbed from the intestine. When fat labelled in various forms, i.e. as elaidic acid (26) or as deuterium marked fat (27), is fed, a large part of the labelled fatty acids can be recovered in the adipose tissue. According to Frazer's 'partition theory' (28) only that part of fat, resorbed as unhydrolyzed glycerides, goes directly to the depots, while fatty acids are absorbed into the portal vein and are transported to the liver. The classical theory, that fat is deposited in the adipose tissue only when given in excess of the caloric requirement, has been finally disproved by the experiments of Schoenheimer and Rittenberg (27) with deuterio-fatty acids. Fifty per cent of the deuterium ingested could be found in the fat stores four days after feeding. This is true even when no change in the total quantity of fat occurs and, as was shown by Bernhard and Steinhauser (29), even when fat disappears from the stores in the fasting mouse. Mobilization and deposition of fat go on continuously, without regard to the nutritional state of the animal. The lowering of the fat content of the tissue during hunger is the result of mobilization exceeding deposition. This can hardly be due to changes in the concentration of fat in the blood, which is on the normal level or even somewhat elevated. In fasting rats treated with phloridzin, the mobilization of fat from the depots is even generally accompanied by a marked elevation in the concentration of blood lipids. The fat level of the blood cannot, therefore, be regarded as the regulator of fat deposition and mobilization.

The balance between deposition and mobilization must thus be controlled by a factor acting directly on the fat cell. In a recent study, we (30) were able to show that the penetration of fat into adipose tissue is an active process, depending upon the metabolism of the cells. Fat-depleted adipose tissue, incubated with serum at 38° C., takes up fat from the medium. This uptake is prevented by heating the tissue to 80°C., by the addition of sodium fluoride and sodium cyanide and by lowering the incubation temperature to 20°C.

b. Fat Synthesized from Carbohydrates and Proteins. The ability of the animal body to build its fat from carbohydrates has been demonstrated in the older literature by balance experiments, and more recently by the recovery of deuterium, given to the animal in the form of deuterium oxide, from the fatty acids in its depots (31). The newly synthesized fat contains mainly C_{16} and C_{18} fatty acids (32). It is generally accepted that this newly synthesized fat is formed in the liver and that no synthesis occurs in adipose tissue itself. Quantitative data, obtained by Schoenheimer and Rittenberg (31) and by Stetten and Grail (33), on the velocity of exchange of fat in the liver and in the depots are very difficult to reconcile with this assumption.

From the results of Stetten and Grail it has been calculated that the actual rate of synthesis of fatty acids by a group of 5 mice was in the neighborhood of one gm/day. This is about four times as much as was present in the livers of the normal mice. If the liver be assumed to be the major site of synthesis, the half life time of fatty acids in the liver becomes a matter of hours and not of days. Actually the half life time of fatty acids in the liver was found to be between 2.6 to 2.8 days and that in the carcass between 5 to 6 days.

The studies of Tepperman, Brobeck and Long (34) suggest that the conversion of carbohydrates into fat may take place both in the liver and in the extrahepatic tissues. These workers compared rats which were trained to eat their entire daily ration in one hour with control animals. Since the trained animals exhibited a mean R.Q. of 1.22 after the administration of glucose, whereas the untreated ones showed, under these conditions, a R.Q. of about 1.05, it was concluded that one effect of the dietary training was an augmentation of the rate of fatty acid synthesis from carbohydrates. The difference in R.Q. between the two groups persisted even in the functional absence of the liver, though it was not so striking as in the intact animal.

The correlation between the appearance of glycogen in adipose tissue and the deposition of fat led Tuerkischer and Wertheimer (35) to suggest that adipose tissue itself is capable of converting carbohydrates into fat. This suggestion is corroborated by the finding that adipose tissue, containing glycogen, exhibits a respiratory quotient exceeding 1.0 (36, 37). Recently, we (38) were able to show that adipose tissue, incubated *in vitro* with serum enriched with deuterium oxide, contains fatty acids with stably bound deuterium. It thus seems established that adipose tissue participates in the synthesis of fatty acids in the body.

DEPOSITION OF GLYCOGEN IN ADIPOSE TISSUE

The presence of glycogen in adipose tissue was first observed in histological studies by v. Gierke (39). Hoffmann and Wertheimer (40), Wertheimer (41) and Tuerkischer and Wertheimer (35) showed that glycogen cannot be demonstrated by chemical methods in normally fed healthy dogs and rats. When, however, the animals were fasted or undernourished and afterwards placed for a short time on a carbohydrate rich diet, glycogen could be found regularly in adipose tissue. This glycogen deposition is transient. The glycogen disappears after a certain time if feeding is continued. The conditions favoring glycogen deposition are generally the same as those enhancing fat deposition. Glycogen deposition precedes fat deposition and comes to an end with the completion of fat deposition (35). Glycogen deposition has been established (35) in the following cases, in addition to refeeding after undernutrition: *a*) in rats trained to consume their entire daily ration in one hour (34); *b*) in rats fed and starved on alternate days (42, 43); *c*) in rats after a certain time of excessive carbohydrate consumption (35); *d*) in rats after injection of insulin in appropriate doses (45); *e*) in new-born rats (immediately after birth the glycogen content of presumptive adipose tissue decreases concurrently with the accumulation of fat, 44); and *f*) in rats after suture of the nerves supplying adipose tissue (45).

The deposition of glycogen is most marked in the interscapular brown fat tissue of the rat. The amount of glycogen deposited in this tissue under suitable conditions

is of the same order of magnitude as that in the liver. Comparatively high quantities are found in mesenteric fat as well. Groin fat and perinephric fat generally contain the smallest quantities of glycogen.

According to the view that adipose tissue is a quasi dead reserve material, the glycogen deposited must be assumed to reach the tissue via the blood stream. The glycogen would then have to diffuse through the cell membrane, as well preserved adipose tissue preparations can be shown to contain glycogen within the cells but not in the intercellular spaces. This is highly improbable; a more plausible explanation is that glycogen is synthesized in the adipose cells themselves.

It has been shown that glycogen accumulation occurs only under suitable conditions and that it is always correlated with an enhanced fat deposition. It thus seems probable that glycogen is directly concerned with fat synthesis in adipose tissue, possibly as an intermediary product in the transformation of carbohydrates into fat. This would explain why glycogen appears only when this synthetic process is especially active.

Adipose tissue cannot be regarded as an additional carbohydrate store for replacing blood sugar, as the findings of Mirski show that no glucose is formed upon breakdown of adipose glycogen (37). The conclusion that adipose glycogen is transformed into fat is corroborated by the high respiratory quotient of glycogen-containing adipose tissue.

REGULATION OF FAT AND GLYCOGEN DEPOSITION AND MOBILIZATION

In the regulation of fat metabolism, liponeogenesis must play as important a rôle as gluconeogenesis in carbohydrate metabolism. This is especially so, since the diet of most animals consists mainly of carbohydrates and fat constitutes the main reserve material of the body. The conversion of food carbohydrates into stored fat goes on continuously. The older view, that fat formation from carbohydrates commences only after the glycogen stores are fully loaded, can no longer be held (31). According to Stetten (46), in the normal rat 30 per cent of the carbohydrates fed are stored as fat and only about 3 per cent as glycogen. In view of the fact that adipose tissue is the main site of fat storage and has been shown to be an actively metabolizing tissue, with a comparatively dense vascular and nervous supply, it is presumable that nervous and endocrine factors influence the deposition and mobilization of fat.

Nervous Regulation

The first observations, indicating nervous regulation of fat metabolism, came from clinical sources. Goering (13), summarizing clinical experience, came to the conclusion that excessive nervous irritation brings about fat loss, while paralysis causes deposition of fat. Mansfeld-Mueller (47) found that upon scission of the femural nerve the denervated side becomes fatter than the intact one. Wertheimer (14) showed that scission of the spinal cord, above the sixth thoracic segment, prevents the formation of fatty liver in phloridzin-treated fasting dogs. Scission below this region allows the fatty infiltration of the liver to proceed. Scission of the liver nerves does not prevent fat infiltration. It thus seems presumable that, in the absence of nervous irritation of the fat tissue, fat mobilization is inhibited. This

conclusion was corroborated by Erben and Hasselbach (48) in experiments with phosphorus poisoned rats and by Mill (49) in experiments of fat mobilization following hemorrhagia. Hausberger (50) was able to cut the nerve connections supplying the symmetrical interscapular fat body of the mouse on one side only, thus setting up conditions in which the normally innervated side of this bilateral symmetrical body served as an ideal control for the otherwise identically situated but denervated other side. Influx of glycogen and, in its wake, an accumulation of fat were noticeable in the denervated side of the interscapular fat body some 10 hours after denervation. The fat content of the denervated side exceeded that of the control side in the normally fed as well as in the starved mouse. The denervated side was depleted of its fat only after long periods of hunger. Beznak and Harris (51) showed, by scission of the splanchnicus, that sympathetic nerve fibers are involved in the mobilization and deposition of fat in the perinephric adipose tissue. Sympathetic control is also shown by the occurrence of facial hemiatrophy on irritation of sympathetic trophic fibers (13). The trophic influence of the autonomous nervous system on fat tissue was also shown by Kuré *et al.* (52) by isolated scission of the sympathicus and of the parasympathicus in dogs. The sympathicus was found to act as an inhibitor of fat deposition and the parasympathicus as an accelerator. After complete denervation, both fat deposition and fat mobilization were reduced. The denervated fat tissue was found to possess a reduced metabolism.

It seems well established that obesity can constantly be induced in rats by lesions of the hypothalamus (53, 54). It is presumed that the neurones involved in the disturbance lie in the region of the ventromedial nuclei and that their axons run into the region above and lateral to the mammillary bodies and from there into the mesencephalic tegmentum (54). From this center, fibers may enter the spinal cord and from there innervate the fat tissue by sympathetic nerve fibers, which leave the spinal cord mainly above the sixth thoracic segment.

Normal innervation seems to be requisite for the attainment of a dynamic equilibrium in fat tissue. Secession of the innervation is followed by elevation in the fat content of the tissue. The nervous influence on adipose tissue may be of high importance in the etiology of obesity and leanness, as well as in localized occurrence of fattening. Adipose tissue seems to possess a certain 'tonus' due to constant innervation. This 'tonus' may be either lowered or raised by pathological conditions.

Endocrine Regulation

1. *Anterior pituitary.* The regulation of fat mobilization from adipose tissue by the pituitary has been proved by Barett, Best and Ridout (55) by the use of deuterio-fatty acids and by Stetten and Salcedo (56) by labelling the newly synthesized fatty acids with deuterium from the body water enriched with heavy water. Both groups found that the fatty livers, produced by the injection of anterior pituitary extracts into fasting mice, are the result of excessive mobilization of depot fat and its migration to the liver. These findings suggest a direct influence of anterior pituitary hormones on adipose tissue activity. The hormone concerned has not been clearly defined. Weil and Stetten (57) showed that the urine of fasting rabbits contains a factor, which acts similarly to the pituitary factor, causing fatty liver upon

injection into a fasting mouse. Depletion of the fat depots and replacement of the fat by a translucent gelatineous material was observed by Dobyns (58) upon injection of a preparation of thyreotropic hormone of the pituitary. The changes were found to take place even in the absence of the thyroid, showing that it was not the thyreotropic hormone proper that caused fat depletion, but another factor present in the preparation. Recently it has been shown by Reiss (59) that lactogenic hormone also reduces the fat content of fat tissue in rats and in man. This mobilization of fat may be connected with lactation. Clement showed that scission of the nerves supplying the interscapular fat body and the perinephric fat abolished the fat mobilizing effect of pituitary extracts (60). The same method was used to prove the dependence of the adrenaline effect on fat mobilization upon the sympathetic system (61).

2. *Adrenal cortex.* The observation of Verzar and Laszt (62), that the deposition of fat in the liver of rats is inhibited in adrenalectomized animals, has been repeatedly confirmed (63–66). All experimental methods used to obtain fatty livers were less successful in adrenalectomized rats. Recently, Hartman, Brownell and Thatcher (67) succeeded in isolating a new factor from cortical extracts, distinct from the sodium and carbohydrate factors, which causes deposition of fat in the liver of starved adrenalectomized animals. The defect in adrenalectomized animals is generally attributed to inadequate mobilization of the fat depots. However, if the lack of fat mobilization were the main factor in the disturbances of fat metabolism in adrenalectomized animals, the fat depots would be rich in fat. Tuerkischer and Wertheimer (35) found that fat deposition in adrenalectomized rats is also markedly below normal. Together with the lack of fat deposition, only negligible amounts of glycogen are deposited, under conditions normally causing a marked deposition of glycogen. Glycogen deposition could be induced, under these conditions, by the administration of fresh cattle adrenal preparations and by large doses of desoxycorticosterone acetate (2 mg.). That the loss of body fat in adrenalectomized rats is not due to reduced food intake was shown by Schiffer and Wertheimer (78) in paired-fed rats, in which the normal always markedly exceeded the adrenalectomized rat in its fat content. Administration of 2 mg. of DCA or of adrenal cortical extracts reestablished fat deposition. These experiments suggest a direct influence of cortical hormones on the fat tissue and on the conversion of carbohydrate into fat. The lack of fatty livers in adrenalectomized rats may partly be due to this disturbance in fat synthesis. Another explanation points to the inability of the liver of adrenalectomized animals to store fat or to a higher fat metabolism in the liver. MacKay (65) showed that adrenalectomized rats store less fat in the liver than normal rats, upon ingestion of cream or pure fat. When adrenalectomy is performed after the establishment of fatty liver, the fat leaves the liver more quickly than in normal rats.

It may be concluded that in adrenalectomized animals, fat deposition in both the liver and the depots is inhibited.

3. *Insulin.* Insulin is known to prevent fatty livers occurring in cases of pancreas or phloridzin diabetes (69). It has recently been shown that insulin also partially inhibits fatty liver production, which occurs in response to the administration of anterior pituitary extracts in fasting rats, mice and guinea pigs (70). It is generally taken for granted that this insulin effect is indirect, acting through the influence of

insulin on carbohydrate metabolism. It has been found by Wertheimer (43) that, under suitable conditions, insulin injection brings about an appearance of glycogen in adipose tissue of the normally fed rat. Glycogen disappears from fat tissue simultaneously with the disappearance of the insulin effect on blood sugar. This insulin effect is not due to the increased appetite induced by insulin, since glycogen deposition in intrascapulary fat precedes the augmentation of appetite. Insulin induces glycogen deposition in intrascapulary adipose tissue even in hunger. However, no glycogen deposition is induced by insulin in rats on a protein rich diet. In adrenalectomized rats, insulin has no effect on appetite, but renews the capacity of adipose tissue to store glycogen. It may be concluded therefore that insulin exerts a direct influence on glycogen synthesis in adipose tissue. Alloxan diabetic rats are unable to store glycogen in their adipose tissue, under conditions which in normal rats regularly bring about such a deposition. Administration of insulin fully restores this ability in alloxan diabetic rats (71).

Experiments of Pauls and Drury (72) as well as those of Stetten and Kleiner (73) proved the deficiency in fat synthesizing capacity of diabetic animals. According to the latter authors, only 5 per cent of the normal amount of carbohydrate is converted into fat in the diabetic animal. Since adipose tissue participates in fat synthesis in the body (38) and insulin is obligatory for normal glycogen and fat deposition in adipose tissue, the obstruction of metabolism in this tissue may be of importance in the pathology of diabetes.

4. *Thyroidea.* Adipose tissue glycogen deposition is enhanced in thyreotoxic rats during the first days of recovery feeding (35). After this period, the glycogen values fall rapidly to zero, whereas in normal rats the maximum glycogen levels are attained only on the second day of recovery feeding. The consumption of glycogen in adipose tissue is enhanced in thyreotoxic rats, *in vitro* as well as *in vivo*. The influence of thyroxin on fat tissue seems to be due to a generally accelerated metabolism.

MacKay and Sherril (74) reported that thyroidectomy in the adult rat, fed with a fat rich diet, causes a marked reduction in body fat content. The animals do not appear less plump nor do they weigh appreciably less than the controls. This is contrary to the general assumption that thyroidectomy should cause fattening. However, in no case could obesity be induced experimentally by thyroidectomy.

METABOLISM OF ADIPOSE TISSUE IN VITRO

Respiration and Respiratory Quotient

The respiratory activity of adipose tissue was measured by Fleischmann (75) and by Scoz (76). The latter author found respiratory quotients of approximately 1.0 for normal adipose tissue, as well as for adipose tissue from fasting animals. High R.Q. values were also frequently found by Ruska and Quast (77). Henle and Szpingier (78), on the other hand, found R.Q. values of 0.75 for normal fat tissue. Mirski (37) showed that the respiratory rate and the respiratory quotient depend upon the condition of the tissue and upon the medium. With fat tissue from fasting rats, suspended in phosphate Ringer's solution, an average R.Q. of 0.64 and an oxygen consumption Q_{O_2} (mm^3 O_2/hr./mg. of fresh tissue) of 0.12 were found. Fat tissue

containing glycogen, obtained after recovery feeding, showed an average R.Q. of 1.05 and a Q_{O_2} of 0.18. The heightened oxygen consumption and the high R.Q. were transient under these conditions and tended to fall after one to two hours. Addition of glucose to hunger fat raised the R.Q. to 0.73. When the milieu was serum, instead of phosphate Ringer's solution, the Q_{O_2} and R.Q. values of hunger fat were 0.18 and 0.78 respectively. With added glucose, the corresponding values were 0.21 and 1.15. In fat tissues containing glycogen, the R.Q. values exceeded 1.0 (av. 1.27) in all cases and the Q_{O_2} value was 0.38. The high R.Q. was maintained for many hours and in some cases rose to 1.6. These experiments lend additional proof for the conversion in adipose tissue of glycogen into fatty acids (38).

Hook and Barron (79) measured the respiration of brown adipose tissue of the hibernating and non-hibernating ground squirrel. They calculated their results on the basis of fat free tissue and found a Q_{O_2} of 17.1, i.e. of the same order as that of kidney tissue. During hibernation, adipose tissue retained a large proportion of its chemical activity at low temperature (3° C.). Respiration was 36 per cent of the value for normal temperature, as against a residue of 15 per cent in kidney and liver. The R.Q. found was 0.80 and fell after starvation for eight weeks to 0.67.

Enzymatic Activity

The presence of diastase, phosphatase, lipase and dehydrogenases in adipose tissue has been repeatedly reported (80). However, the demonstration of diastatic or phosphatatic activity in extracts of adipose tissue does not serve as conclusive proof for the presence of the enzymes in the tissue itself, since they may be derived from the blood included in the tissue. Mirski (37) was able to show that 'adipose diastase' is similar in its properties to blood diastase, converting glycogen into low polysaccharides which are not fermentable. Only 5 per cent of the glycogen broken down was accounted for as glucose. Hausberger (80), on the other hand, tried to prove that diastase and phosphatase originate in the tissue itself, by showing that the first enzyme is concentrated in denervated adipose tissue and that the activity of the second is enhanced on recovery feeding. An alternative path for glycogen decomposition in adipose tissue was demonstrated by Mirski (37), who showed that adipose tissue phosphorylates glycogen. The product of phosphorolysis is Cori-ester. No phosphoglucomutase was found in white adipose tissue. Brown adipose tissue, on the other hand, was found to contain this enzyme. The lack of glucomutase in white adipose tissue leaves the mechanism of glycogen synthesis in this tissue unexplained.

Among the various dehydrogenases present in adipose tissue, special interest pertains to the dehydrogenases of higher fatty acids. Their presence in adipose tissue was repeatedly demonstrated (20–23). According to Yosii (21) adipose tissue is the richest source of dehydrogenase of the higher fatty acids, exceeding the liver 2 to 3 times and the muscle and kidney up to 30 times. Shapiro and Wertheimer (22) showed that dialysed water extracts of adipose tissue dehydrogenate fatty acids only in the presence of phosphate and a boiled extract of adipose tissue or yeast. In agreement with Lang and Mayer (81), it was found that the co-factor present in the boiled extracts can be replaced by adenosine-5-phosphoric acid. The enzyme, studied by Lang and Mayer in liver extracts and by Shapiro and Wertheimer in adipose tissue,

seems to be different from other fatty acid dehydrogenase found (82), serving only for interconversions of fatty acids and not for their total breakdown.

Other substrates found to be attacked by adipose tissue extracts are phospholipids, succinic acid, glycerophosphoric acid and lactic acid (22).

BROWN ADIPOSE TISSUE

The brown adipose tissue is in many of its morphological and physiological properties quite distinct from ordinary, white, adipose tissue (83). It shows a much higher respiratory and metabolic activity than white adipose tissue. Under appropriate conditions, brown adipose tissue is capable of storing glycogen in concentrations comparable with those in the liver (35). On the other hand, fat mobilization from brown adipose tissue during hunger is much slower than that from white fat tissue (84). Due to these structural and metabolic peculiarities, various special functions have been ascribed to brown adipose tissue. Since the interscapular brown fat body is especially developed in hibernating animals, this tissue has been connected with hibernation and has been named 'hibernating gland'. The older literature on this aspect can be found in a review by Ferdmann and Feinschmidt (85) and in an article by Rasmussen (86). In the more recent literature two reports can be found that bring evidence pointing to a function of brown adipose tissue in hibernation. Wendt (87) showed that the injection of extracts of brown fat of the hibernating hedgehog into rats lowered the metabolism of the rats by 20 to 30 per cent. After an initial unrest, the animals became quiet and apathetic. Similar results were obtained by Hook (88) who injected white rats with an extract of brown fat of woodchucks and ground squirrels. Extracts of fat from the omentum or of perinephric fat tissue had no such influence. No experiments were made with extracts of brown fat of non-hibernating animals. The experiments prove only the presence in brown fat of a fat soluble substance that depresses the metabolism of the white rat.

In man and in the rabbit, the brown adipose tissue exists only in the embryo. In the rat and in the mouse, the brown fat in its embryonic form remains at various sites during adult life. White fat tissue, after depletion of most of its fat, becomes morphologically and functionally very similar to brown fat tissue. Brown fat tissue may therefore be looked upon as an adipose tissue that did not develop beyond its embryonic state.

RÔLE OF ADIPOSE TISSUE IN THE DEVELOPMENT OF OBESITY

As in other fields of physiology, much may be learned about the normal function of a tissue from the elucidation of disturbances during pathological conditions. The state of knowledge about experimental obesity has been recently reviewed (89, 53, 54). However, the place of adipose tissue in these disturbances has received very little attention. In one case, of congenitally obese mice, it could be clearly demonstrated by Salcedo and Stetten (90) that the retardation of fat mobilization from the depots is an important factor in the pathology of obesity. The mice were fed for five days on bread crumbs, to which ethyl esters of deuterio-fatty acids were added. Analysis of the deuterium content of body water and fatty acids showed that the storage of fatty acids, in these mice, was normal. The turnover of depot fatty acids, however,

was found to be considerably lower than in normal animals. A similar study, in the case of the best studied form of experimental obesity, in hypothalamic obesity (53, 54) would be of great interest.

CONCLUSIONS

Adipose tissue is a tissue with a special structure and a special type of cell. It is supplied by a comparatively dense capillary net and innervated by sympathetic nerve fibers. Deposition and mobilization of fat in adipose tissue is an active process, involving the metabolism of the tissue. Under conditions favoring fat deposition, adipose tissue accumulates glycogen, which is presumably built in the tissue cells themselves. Synthesis of new fatty acids from carbohydrates as well as transformation of one fatty acid into another proceed continuously in this tissue. All of these metabolic activities are regulated by nervous and endocrine factors.

The present knowledge points to the possible rôle played by adipose tissue in the development of various pathological conditions in the metabolism of the body.

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INDEX

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PHYSIOLOGICAL REVIEWS

Contents of Volume 27, 1947

AUGUST PI-SUÑER: The Regulation of the Respiratory Movements by Peripheral Chemo-Receptors
J. P. BOUCKAERT AND CHR. DE DUVE: The Action of Insulin
MAX S. DUNN, EDWARD A. MURPHY AND LOUIS B. ROCKLAND: Optimal Growth of the Rat
FREDERICK L. HISAW: Development of the Graafian Follicle and Ovulation
HARRY GOLDBLATT: The Renal Origin of Hypertension
HAROLD G. WOLFF AND JAMES D. HARDY: On the Nature of Pain
WILLARD MACHLE AND T. F. HATCH: Heat: Man's Exchanges and Physiological Responses
A. G. OGSTON: The Definition and Meaning of pH
FRANK A. BEACH: A Review of Physiological and Psychological Studies of Sexual Behavior in Mammals
D. W. WOOLLEY: Recent Advances in the Study of Biological Competition Between Structurally Related Compounds
KARL MEYER: The Biological Significance of Hyaluronic Acid and Hyaluronidase
H. R. CATCHPOLE AND ISIDORE GERSH: Pathogenetic Factors and Pathological Consequences of Decompression Sickness
LOUIS N. KATZ: The Genesis of the Electrocardiogram
ROBERT CHAMBERS AND B. W. ZWEIFACH: Intercellular Cement and Capillary Permeability
A. R. MCINTYRE: Some Physiological Effects of Curare and Their Application to Clinical Medicine
CECIL JAMES WATSON AND EVREL A. LARSON: The Urinary Coproporphyrins in Health and Disease
MAX KLEIBER: Body Size and Metabolic Rate
EARL R. LOEW: Pharmacology of Antihistamine Compounds
T. H. BULLOCK: Problems in Invertebrate Electrophysiology
JOHN A. LUETSCHER, JR.: Biological and Medical Applications of Electrophoresis
KONRAD BLOCH: The Metabolism of Acetic Acid in Animal Tissues

PHYSIOLOGICAL REVIEWS

Tentative Contents of Volume 28, 1948 (*Continued*)

E. Ball and E. A. Evans, Jr.: Biochemistry of the Malaria Parasite

Thomas Francis, Jr.: Principles and Problems in Virus Infection

R. D. H. Heard: The Origin and Significance of Urinary 17-Keto-Steroids

J. O. Hutchens and M. A. Lipton: Cyanide and Cyanogen Chloride

N. Kleitman: Some Physiological Rhythms

Bradley M. Patten: Initiation of Cardiac Contraction in Embryos

R. A. Peters: The BAL Treatment of Metal Poisoning

H. P. Smith: Blood Clotting Mechanisms

Van S. Smith and O. Watkins Smith: Internal Secretions and Toxemia of Late Pregnancy

S. Spiegelman: Mechanisms Controlling Cellular Enzymatic Constitution

A. E. Wilhelmi: The Phosphatases

E. M. Landis and John Hortenstine: The Functional Significance of Venous Blood Pressures

T. H. Jukes and E. R. Stokstad: Pteroylglutamic Acid and Related Compounds

Benjamin H. Ershoff: Conditioning Factors in Nutritional Disease

J. W. Beard: The Chemical, Physical and Morphological Properties of Animal Viruses

R. W. Gerard and J. M. Tobias: The Mechanism of Phosgene Poisoning

Albert Tyler: Fertilization and Immunity

F. D. W. Lukens: Experimental Diabetes

E. E. Snell: Use of Micro-Organisms for Assay of Vitamins

F. W. Hartman: Liver Injury

V. G. Dethier and L. E. Chadwick: Chemoreception in Insects

Ussing: Transport of Ions Across Cellular Membrane

J. E. P. Toman and L. S. Goodman: Anticonvulsants

Max Dunn: Analysis of Amino Acids by Microbiological Technique

Shear: Tumor Inhibitors

E. Wertheimer: Physiology of Adipose Tissue

G. Medes: Sulfur Metabolism

J. C. Eccles: Excitation and Inhibition

Kreyberg: Frostbite

J. D. Bernal: Molecular and Macromolecular Structure in the Interpretation of Physiological Processes

Henry Borsook: Synthesis of Proteins

Leigh Hoadley: Developmental Physiology

Mary E. Rawles: Physiology of Pigmentation in Vertebrates

J. Percy Baumberger: Oxidation-Reduction Potential in the Interpretation of Physiological Processes

G. W. Harris: Neural Control of the Pituitary Gland

PHYSIOLOGICAL REVIEWS

Contents of Volume 24, 1944

David P. C. Lloyd: Functional Organization of the Spinal Cord

L. H. Newburgh: Obesity. I. Energy Metabolism

J. W. Conn: Obesity. II. Etiological Aspects

Edwin E. Osgood and Arthur J. Seaman: The Cellular Composition of Normal Bone Marrow as Obtained by Sternal Puncture

Alan R. Moritz: Chemical Methods for the Determination of Death by Drowning

W. W. Swingle and J. W. Remington: The Rôle of the Adrenal Cortex in Physiological Processes

E. W. McHenry and Jean M. Patterson: Lipotropic Factors

Joseph C. Bock: The Benign Meliturias

Harold P. Rusch: Extrinsic Factors that Influence Carcinogenesis

Hans Popper: Distribution of Vitamin A in Tissue as Visualized by Fluorescence Microscopy

Shields Warren. The Histopathology of Radiation Lesions

Samuel Lepkovsky: The Bread Problem in War and in Peace

Donald H. Barron: The Changes in the Fetal Circulation at Birth

Armand J. Quick: The Anticoagulants Effective in Vivo with Special Reference to Heparin and Dicumarol

Julius H. Comroe, Jr.: The Hyperpnea of Muscular Exercise

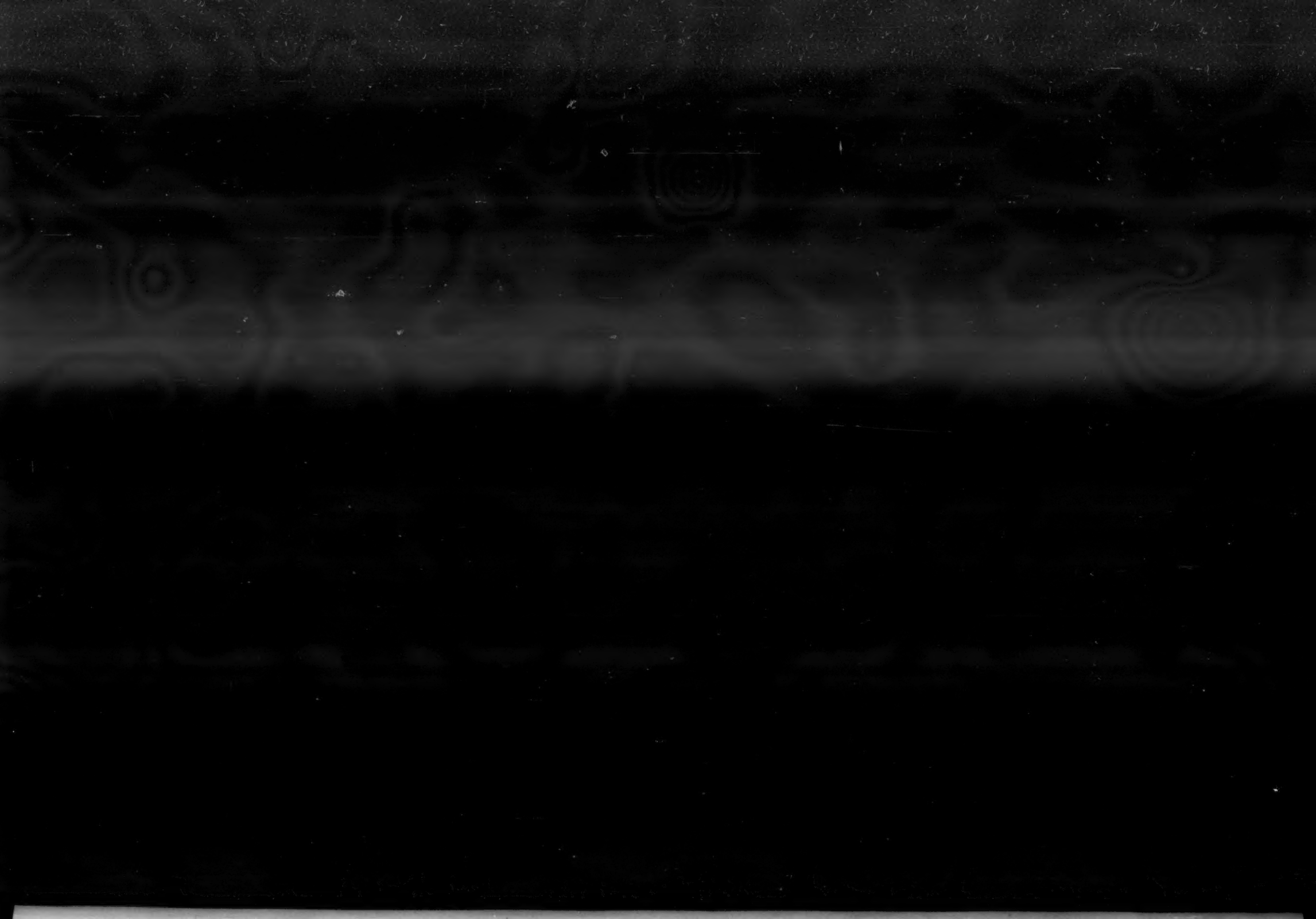
W. E. Petersen: Lactation

Robert Elman: Maintenance of Nitrogen Balance by the Intravenous Administration of Plasma Proteins and Protein Hydrolysates

Warren S. McCulloch: The Functional Organization of the Cerebral Cortex

R. E. Haist: Factors Affecting the Insulin Content of the Pancreas

Herluf H. Strandskov: Physiological Aspects of Human Genetics, Five Human Blood Characteristics

Ernst Fischer: Vertebrate Smooth Muscle

John P. Peters: Water Exchange